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Award Number: DAMD17-01-1-0300

TITLE: Tumor Suppression and Sensitization to Taxol Induced Apoptosis of E1A in Breast Cancer Cells

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REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual Summary (1 Jun 03-31 May 04)

4. TITLE AND SUBTITLE
Tumor Suppression and Sensitization to Taxol Induced Apoptosis of E1A in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-01-1-0300

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy.

In trying to understand the mechanism underlying E1A's antitumor activity, we have found that E1A downregulated VEGF expression both *in vitro* and *in vivo*, and mapped the domains required for this activity. We have also identified additional new target genes that were critically involved in E1A–mediated chemosensitization.

Also, we have been trying to identify other molecules that may be regulated by E1A via the genomic and proteomic approaches. These studies can provide useful information for us to better understand the molecular functions of E1A, and hopefully we can use this knowledge to better design a mutant E1A construct for cancer gene therapy in the future.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, E1A, Chemosensitization	99
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy in the future. The successful reconstruction of this mutant E1A gene will largely depend on our in vitro as well as in vivo studies of the different mutant stable cells.

Body

Statement of Work (no change):

Task 1: To test whether E1A could repress VEGF expression and whether or not mutation of the CR1 domain of E1A will abrogate this activity.

Task 2: To test whther CR1 domain of E1A represses VEGF transcription through recruitment of HDAC-1 via binding with p300

Task 3: To test whether E1A promotes apoptosis through its CR2 domain by disruption pRB-E2F-1 complexes, releasing free E2F-1.

Task 4: To test the therapeutic effect of the E1A N-terminal mutant as an alternative construct for gene therapy in a nude mouse model.

A. Studies and Results:

In the past year we have continued to work on understanding the molecular mechanisms underlying E1A's proapoptotic effect and antitumor activity. Also we have been trying to identify other molecules that may be regulated by E1A via the genomic and proteomic approaches. This work

may provide useful information for identification of novel target genes of E1A and new biomarkers as therapeutic targets. The progress for each aim will be discussed separately as following:

Task 1:

We have observed that stable expression of E1A do repress VEGF expression by Northen blot in cell culture *in vitro* (Figure 1B) and by immunohistochemistry in tumor tissue *in vivo* (Figure 1B). We also mapped the domains of E1A responsible for downregualtion of VEGF expression (Figure 1C). We found that deletion mutation of an N-terminal fragment (ΔNT) of E1A enhanced the repression effect of E1A in VEGF expression in animal models (Figure 1C).

Task 2:

Using antibody array approach, we identified quite a few molecules that are associated with E1A (Figure 2). By comparing the antibody array pattern obtained from cell lysates of wild type E1A and different functional domain deletion mutation of E1A, we obtained evidences to support our hypothesis that repression of VEGF expression by E1A is achieved by inhibition of the histone accetyltransferase (HAT) activity of p300 via HDAC1. We found that the mutation of these functional domain do affect E1A's association with several proteins (Figure 3A). In particular, the N-terminal deletion mutant binds with HDAC1 more than wild-type E1A, while deletion of either the N-terminal or CR1 domain did not affect E1A binding with pRB or pRB2. However, deletion mutation of the CR2 domain resulted in a loss of binding with pRB, though its binding with pRB1 was enhanced (Figure 3A). Because wild-type E1A and the CR2 mutant bind HDAC1 to a lesser extent, the binding of E1A with HDAC1 could be through indirect binding with pRB. In addition, to test whether the altered binding with HDAC1 in ΔNT mutant will contribute to its enhanced activity in repressing the expression of VEGF through p300, we transiently transfected wild-type E1A and different domain deletion mutant stable cells with a Flag-tagged HADC1 construct and did a co-IP study by IP with anti-Flag antibody (M2) and Western blot with p300. We found that more p300 were recruited to HDAC1 in ΔNT cells than wild-type E1A or ΔCR1 and ΔCR2 mutant (Figure 3B). These findings consistent with our studies on VEGF expression in animals (Figure 1C) and suggest that repression of VEGF expression by E1A might be achieved by inhibition of the histone acetyltransferase (HAT) activity of p300 through its binding with pRB and recruitment of HDAC1.

Task3:

In the past year, we have been intensively working on the molecular mechanisms underlying E1A's proapoptotic effect. We have not yet been able to test the role of free E2F-1in E1A–mediated chemosensitization, however, report from Dr. Lowe's group did show that free E2F-1 played a critical role in E1A-mediated chemosensitization in normal fibroblasts through upregulation of pro-caspase enzymes, such as caspase-3, -7, -8, and –9 (Nahle Z, et al, Nature Cell Biology, 2002; 4 (11): 859 – 864) (1). However, when we screening our E1A stable cell lines established in human cancer cells with epithelial origin, such as human breast (MCF-7, MDA-MB-231), ovarian (2774, SKOV3.ip1), and prostate cancer cell lines (PC-3), we did not obtain a consensus enhancement of these procaspase enzymes (Figure 4A). In addition, loss of pRB expression in human cancer cells either from epithelial origin or from fibroblast did not result in enhanced expression of these procaspase enzymes (Figure 4B). These results suggest that additional mechanisms may be involved in E1A-mediated sensitization to drug-induced apoptosis in human cancer cells.

Therefore, in addition to the original proposed experiment, we also explored those molecules involved in regulation of apoptosis and we found that E1A downregulated a critical oncogenic survival factor Akt while upregulated a proapoptotic factor p38. We demonstrated that activation of p38 and inactivation of Akt were necessary and sufficient for E1A–mediated sensitization to apoptosis induced by serum-starvation, ultraviolet (UV) -irradiation, tumor necrosis factor (TNF) -α, and different categories of anti-cancer drugs, such as adriamycin/doxorubicin, cisplatin, methotrexate, gemcitabine and paclitaxel (Taxol) (data published in ***MCB***, 2003,23: 6836-48, also see appendix). Further studies show that E1A by upregulation of the catalytic subunit of PP2A (PP2A/C) enhanced the activity of PP2A, which results in repression of Akt activation in E1A expressing cells (Figure 5). In addition, we showed that activation of PP2A/C is required for E1A–mediated sensitization to drug–induced apoptosis, since blocking PP2A/C expression using a specific small interfering RNA (siRNA) against PP2A/C reduced drug sensitivity in E1A-expressing cells. Deletion mutation of the CR2 of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, also abolished E1A's ability to upregulate PP2A/C. A manuscript described the above results have been accepted recently by ***Cancer Research*** as **Advance in Brief** (see appendix).

Recent reports published in Nature Genetics suggested a tumor suppressor role for p38, as inactivation of p38 by a protein phosphatase PPM1D (also called Wip1, which stands for a wild-type p53-inducible protein phosphatase) is involved in the development of human cancers by suppressing p53 activation (2-4). As Wip1 is also located within a breast cancer amplification

epicenter (5), We also wanted to test if E1A could affect Wip1 activity. By screening several pairs of E1A stable cell lines established in human breast cancer, we found that expression of E1A do repress Wip1 protein expression compared with the respective vector transfected control cells (Figure 6). In addition, we also found that repression of Wip1 expression was correlated with E1A-mediated activation of p38 activity and inactivation of Akt (Figure 6). By using small interference RNA (siRNA) approach, we blocked Wip1 expression and observed that repression of Wip1 also resulted in enhanced expression of phosphorylated p38 (Figure 7A) and increased spontaneous apoptosis in MCF-7 cells after blockade of Wip1 by the siRNA (Figure 7B). Therefore, we constructed a set of retroviral vectors expressing stable Wip1 siRNA against various domains of Wip1 and were transfected into MCF-7 cells. Stable cells with reduced levels of Wip1 expression were selected and ready for future characterization. As Wip1 is also located within a breast cancer amplification epicenter (5), we therefore proposed to determine if Akt will directly affect Wip1 phosphatase activity and indirectly affect p38 activation. We found that Akt directly associated with Wip1 (Figure 8A) and phosphorylated Wip1 by in vitro kinase assay using purified recombinant GST-Wip1 as a substrate for Akt (Figure 8B). Further experiments are proposed to determine which site(s) of Wip1 will be phosphorylated by Akt and whether phosphorylation of Wip1 by Akt affects Wip1 phosphatase activity toward its substrates, such as p53 and p38. To better understand the biological function of Wip1 and map the domain(s) interacts with Akt, a serial deletion mutation constructs of Wip1 were established. In addition, to further evaluate the role of Wip1 in mammary tumorigenesis, a mammary specific Wip1 overexpression transgenic model is planned. We will also validate whether Wip1 is a good target for the treatment of human breast cancer by in vitro as well as in vivo studies using the Wip1 transgenic model.

Task 4:

The preliminary results we obtained from the animal model study showed that at least the N-terminal mutant of E1A worked as good as that of the wild-type E1A in terms of repression tumor growth and sensitization to chemotherapy *in vivo* in the context of a systemic gene therapy (Figure 9A). After combined two independent experiments together (N= 8), we monitored the survival rate of wild-type E1A or N-terminal deletion mutant with or without combination with Taxol for more than a year. Again, we observed that the N-terminal mutant of E1A worked as good as that of the wild-type E1A in terms of prolongation of animal survival time (Figure 9B).

Although we are not at the time to construct an alternative E1A construct for gene therapy based on the above results, we have constructed a C-terminal E1A mutant and tested its in vivo effect on tumor

growth in animal models. Since we have not been able to establish a stable cell lines with C-terminal expression, we have constructed a tet-on/off regulated wild–type E1A and C-terminal E1A inducible expression constructs and established tet-off/on inducible cell lines with wild–type E1A or C-terminal E1A expression in breast cancer MCF-7 cells. The biological effects of these stable cells and the therapeutic efficacy of this C-terminal mutant in comparison with wild-type E1A and a N-terminal mutant E1A gene in a gene therapy setting await further analysis.

To gain a global view of genes and proteins regulated by E1A, we also used cDNA microarray and proteomic technologies including 2-D gel electrophoresis and Ciphergen protein chip array to identify the target genes and proteins associated with E1A in breast cancer cells. Differentially expressed genes were found in E1A stable cells versus parental cells by either the cDNA microarray or 2-dimensional gel electrophoresis and Ciphergen protein chip technology (Figure 10). Althoguh these experiments are not directly related to the original proposal, they are relevant to E1A's proapoptotic effect and anti-tumor activity and to breast cancer. Therefore, we will continue to pursue on finding E1A associated molecules and dissect their function on tumor growth and apoptosis in breast cancer.

Key research accomplishments:

(1). We mapped domains of E1A responsible for downregulation of VEGF expression.
(2). Evaluated the chemosensitization effect of E1A by systemic E1A gene therapy approach. (Cancer Gene Therapy, 2004).
(3). We have found that upregulation of p38 activity and downregulation of Akt activity are necessary and sufficient for E1A–mediated sentization to apoptosis induced by serum–starvation, TNF-α, UV-irradiation, and different categories of anticancer drugs (MCB, 2003).
(4). We have found that E1A downregulated a protein phosphatase PPM1D expression, which is amplified/overexpressed in human breast cancer.
(5). We identified that a few novel proteins associated with E1A by antibody array approaches and some of them have been also confirmed by co-IP and western blot analysis.
(6). We have found that upregulation of PP2A activity by E1A contributes to E1A-mediated downregulation of Akt activation and correlates with E1A-mediated chemosensitization (Cancer Research, in press, 2004).
(7). We provided data to support that repression of VEGF expression by E1A might be achieved by inhibition of the HAT activity of p300 through binding with pRB and HDAC1 by antibody array and Co-IP approaches.

(8). We have mapped that CR2 domain of E1A is required for E1A-mediated downregulation of Akt, upregulation of PP2A/C and p38 (MCB, 2003; Cancer Research, in press, 2004).

Reportable outcomes:

1. Manuscripts accepted or published:

   Liao Y, Hung MC: "Regulation of p38 activity by Akt and its association with adenoviral E1A-mediated sensitization to apoptosis. " ***MCB***, 2003, 23: 6836-48.

   Liao Y*, Zou YY, Xia WY, Hung MC: "Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer." ***Cancer Gene Therapy***, 2004: in press.

   Liao Y, Hung MC: " A new role of protein phosphatase 2A in adenoviral E1A-mediated sensitization to anti-cancer drug-induced apoptosis." ***Cancer Reseach***, 2004: in press.

   Xia WY, Chen SJ, Zhou X, Sun PR, Lee DF, **Liao Y**, Zhou BP, Hung MC: Phosphorylation/Cytoplasmic Localization of p21 Cip1/WAF1 Is Associated with HER2/*neu* Overexpression and Provides a Novel Combination Predictor for Poor Prognosis in Breast Cancer Patients. ***Clinical Cancer Research***, 2004; 10:3815-24.

2. Abstracts and presentations:

   Liao Y*, Hung MC: **"Integration of the pro- and anti-apoptotic signals by adenoviral E1A proteins contribute to E1A-mediated sensitization to anti-cancer drug-induced apoptosis in human breast cancer." The 24th IABCR international conference, Sacramento, November 2-6, 2003**

3. Stable cell lines and constructs:

   Stable Wip1 promoter driving luciferase reporter expression construct in MCf-7 cells

   pSuper-Wip1 siRNA expression construct in MCF-7 cells

   Serial deletion mutation constructs of Wip1

Conclusion:

These studies on the molecular mechanisms underlying E1A's tumor suppression and chemosensitization can help us to better design an alternative E1A constructs for future gene therapy. Specifically, identification of additional E1A target genes, such as PPM1D, PP2A, Akt and p38, may help us in finding novel ways to treat cancer patients by targeting the deregulated signals. The combination of E1A gene therapy with Taxol or other chemotherapeutic drugs is one potential new therapeutic approach for the treatment of cancer patients, as we have shown in the animal models. The mutant E1A construct(s), if it works in animal model, could potentially be translated into the clinic and be of great benefit to breast cancer patients.

Reference:

1. Nahle Z, Polakoff J, Davuluri RV, *et al.* Direct coupling of the cell cycle and cell death machinery by E2F. Nature Cell Biology 2002;4: 859-864.
2. Bulavin DV, Phillips, C., Nannenga, B., Timofeev, O., Donehower, L.A., Anderson, C.W., Appella, E., Fornace,A.J., Jr. Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK–mediated activation of the p16Ink4a-p19Arf pathway. Nat. Genet. 2004;36: 343-350.
3. Bulavin DV, Demidov ON, Saito S, *et al.* Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity Nat Genet 2002;31: 210-215.
4. Li J, Yang, Y., Peng, Y., Austin, R.J., van Eyndhoven, W.G., Nguyen, K.C., Gabriele, T., McCurrach, M.E., Marks, J.R., Hoey, T., Lowe, S.W., Powers, S. Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23. Nat. Genet. 2002;31: 133-134.
5. Sinclair CS, Rowley, M., Naderi, A., Couch, F.J. The 17q23 amplicon and breast cancer. Breast Cancer Res. Treat. 2003;78: 313-322.

Appendices:

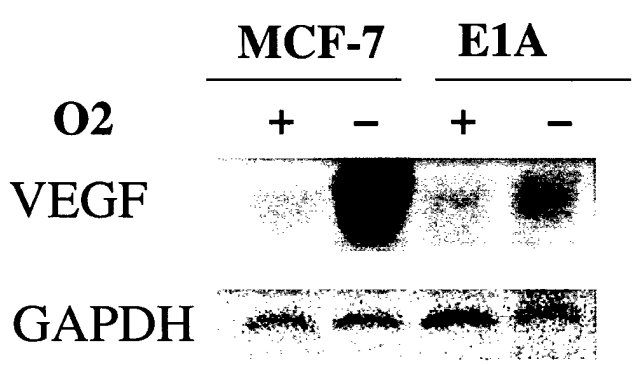
Reprints/manuscripts (MCB, Cancer Res., Cancer Gene Therapy, Clinical Cancer Res.)

Abstract (IABCR)

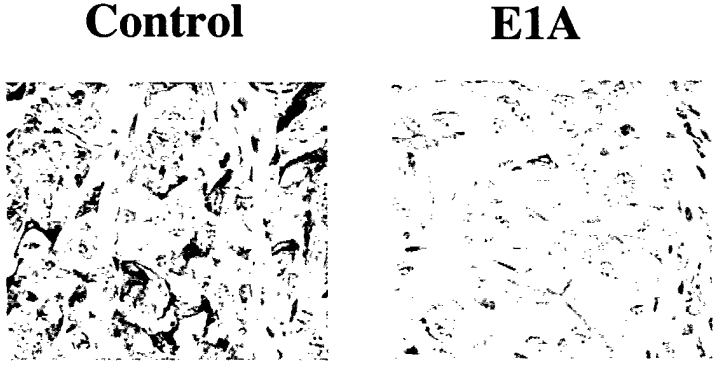
Personal Information (Address, telephone number, Fax, Email)

Figure 1. **E1A downregulate VEGF expression *in vitro* (A) and *in vivo (B-C).***

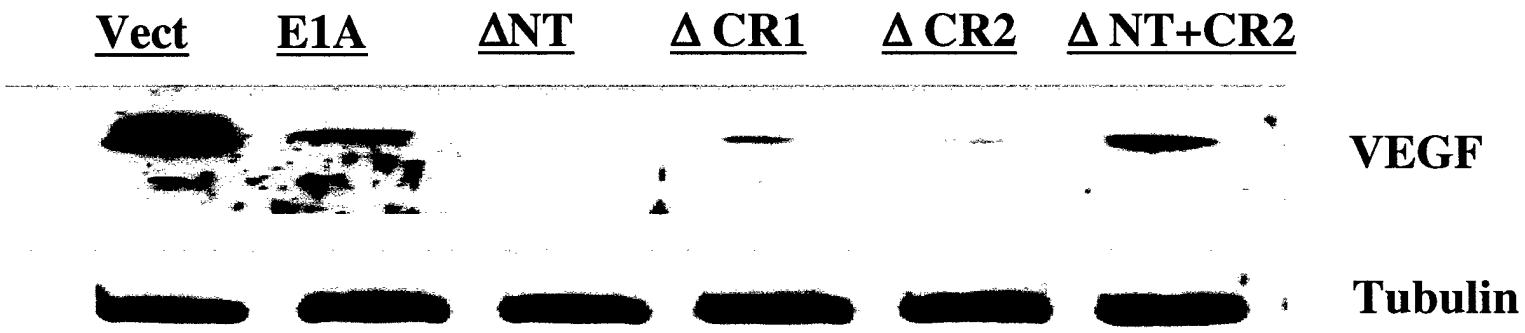
A. Northern Blot analysis of VEGF expression in cell culture *in vitro*.



B. Immunohistochemistry analysis of VEGF expression in tumor tissue *in vivo*.



C. Western blot analysis of VEGF expression in tumor tissues *in vivo* obtained from inoculation of vector control cells or wild-type E1A and different domain mutant E1A stable cells.



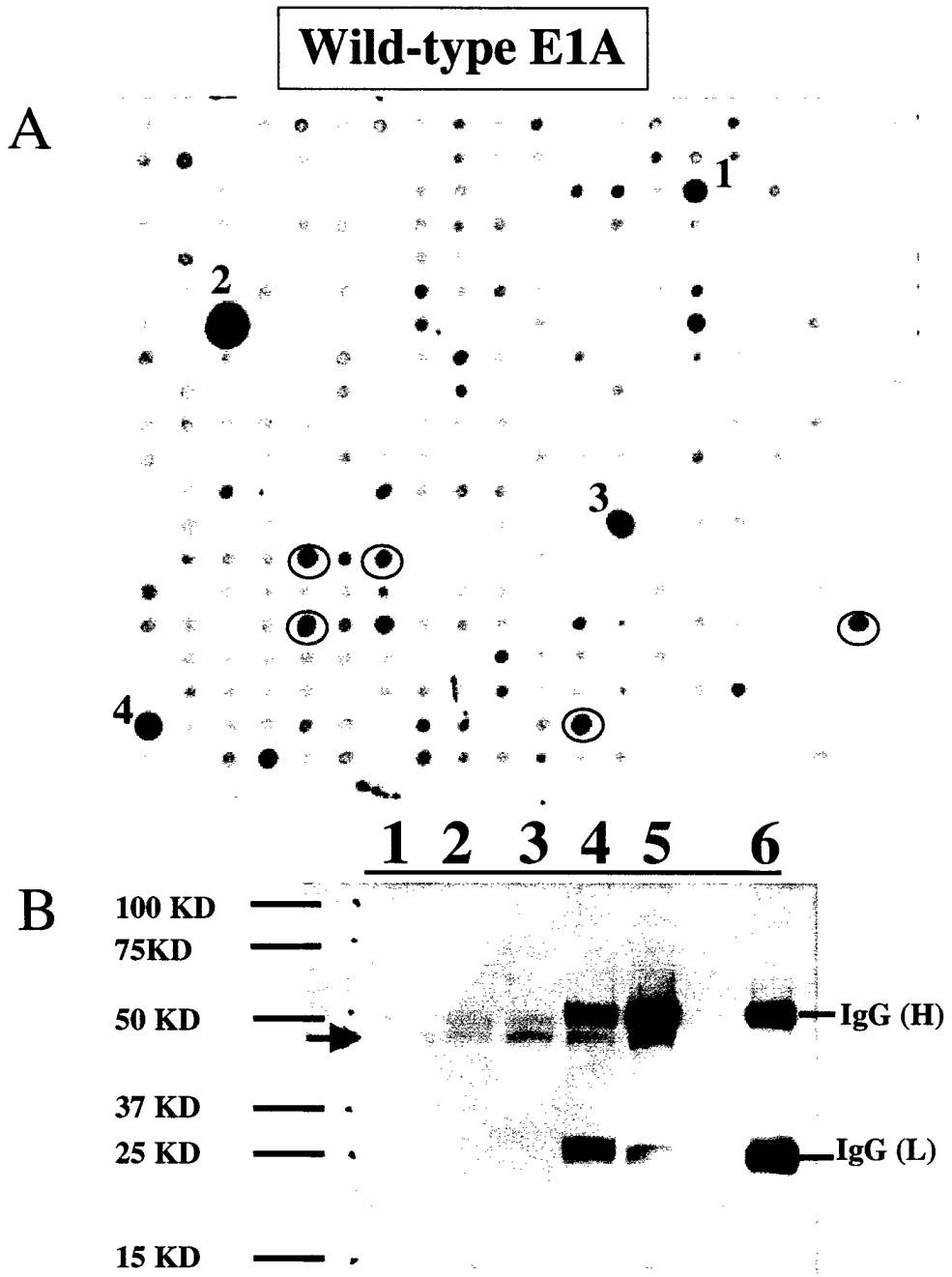
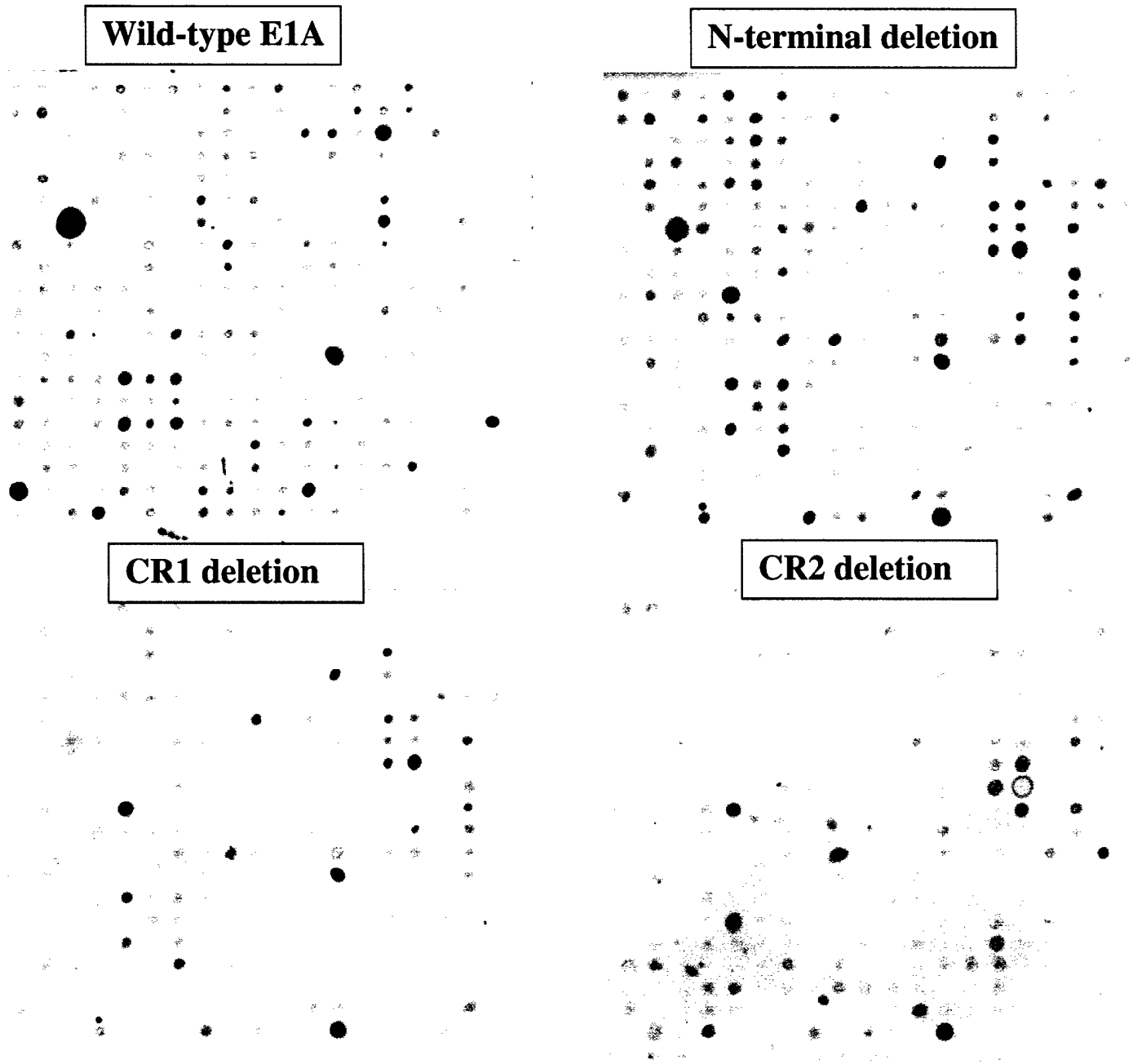


Figure 2

A. Antibody array of proteins associated with E1A. Number 1, 2, 3, and 4 represent proteins that have not been reported to be associated with E1A, but were identify by antibody array and their association with E1A were further supported by co-immuoprecipitation as shown in Figure B. Circled proteins are those previously reported to be associated with E1A.

B. Proteins marked as 1 to 4 in Figure A were immunoprecipitated by respective antibodies and bloted against anti-E1A antibody (M73). Number 5 represents a positive control by using anti-E1A antibody for the immunoprecipitation. Number 6 represents a negative control by using normal IgG. Arrow indicates the E1A protein band.

C


Wild-type E1A
N-terminal deletion
CR1 deletion
CR2 deletion

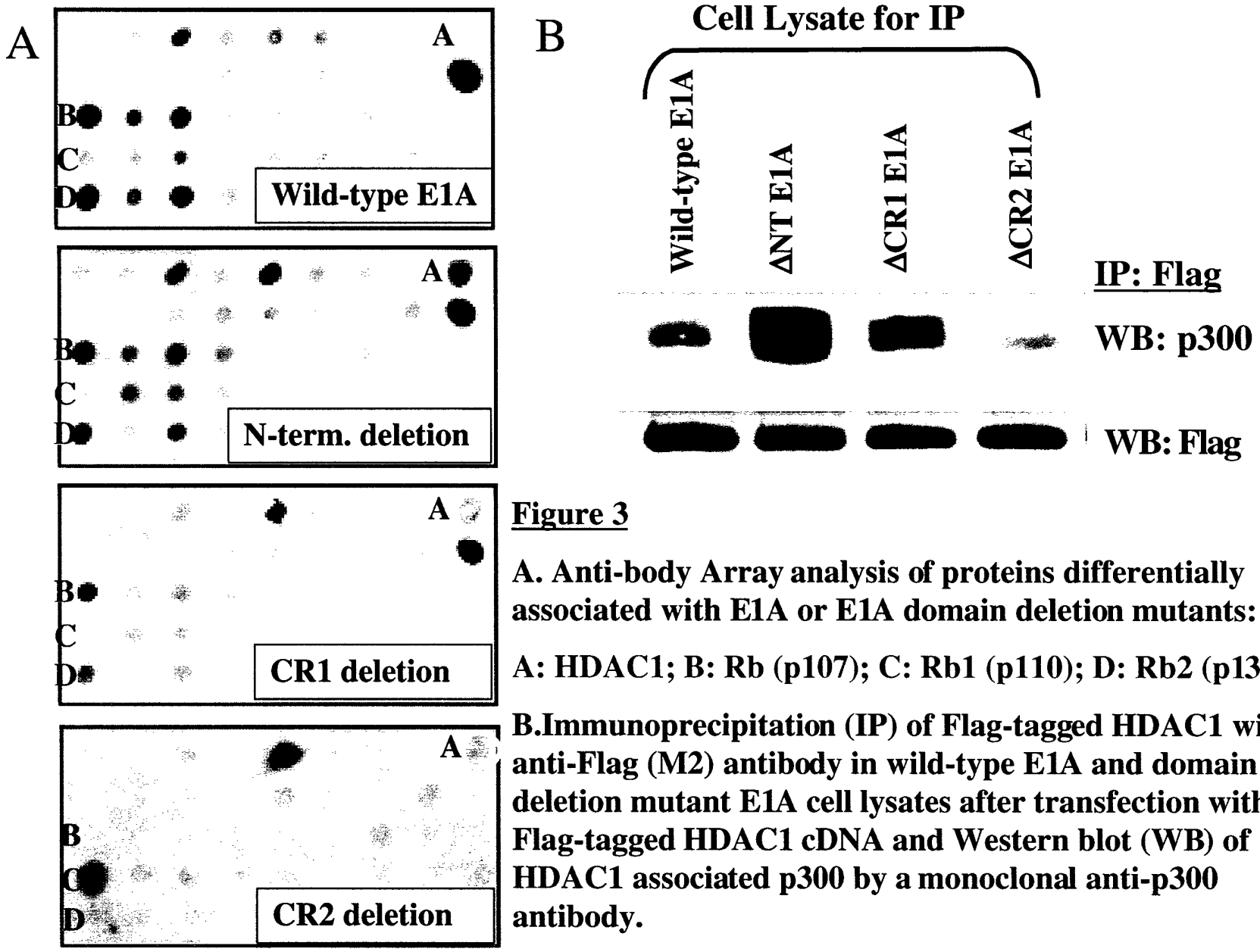


Figure 3

A. Anti-body Array analysis of proteins differentially associated with E1A or E1A domain deletion mutants:

A: HDAC1; B: Rb (p107); C: Rb1 (p110); D: Rb2 (p130)

B.Immunoprecipitation (IP) of Flag-tagged HDAC1 with anti-Flag (M2) antibody in wild-type E1A and domain deletion mutant E1A cell lysates after transfection with Flag-tagged HDAC1 cDNA and Western blot (WB) of HDAC1 associated p300 by a monoclonal anti-p300 antibody.

A

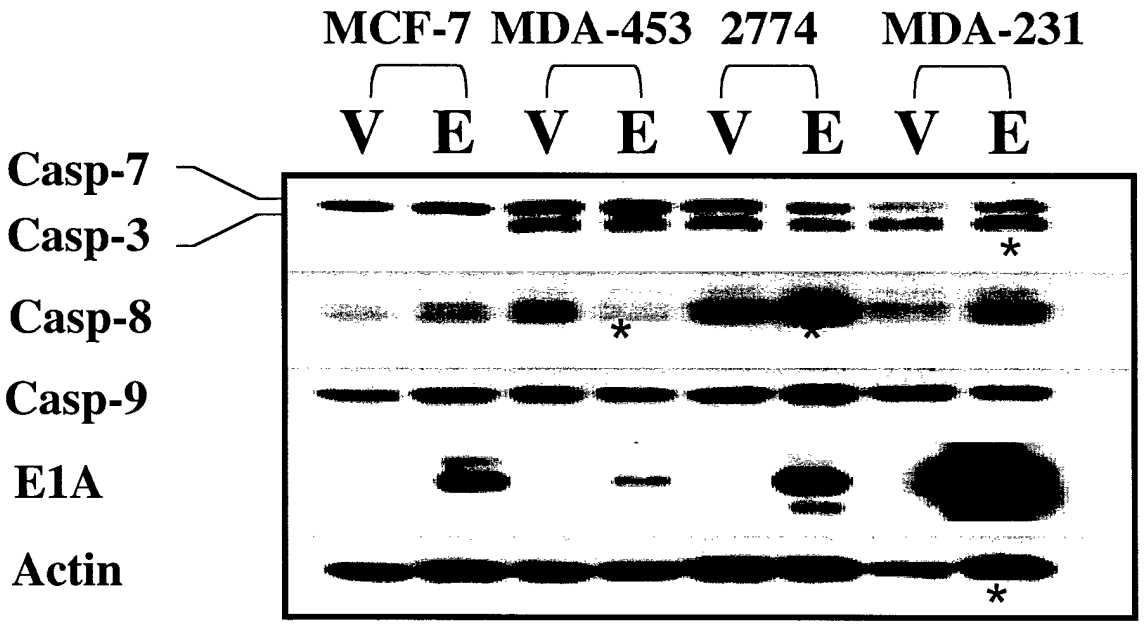


Figure 4

A. Expression of E1A in human carcinoma cells may not results in transcriptional upregulation of caspase proenzymes

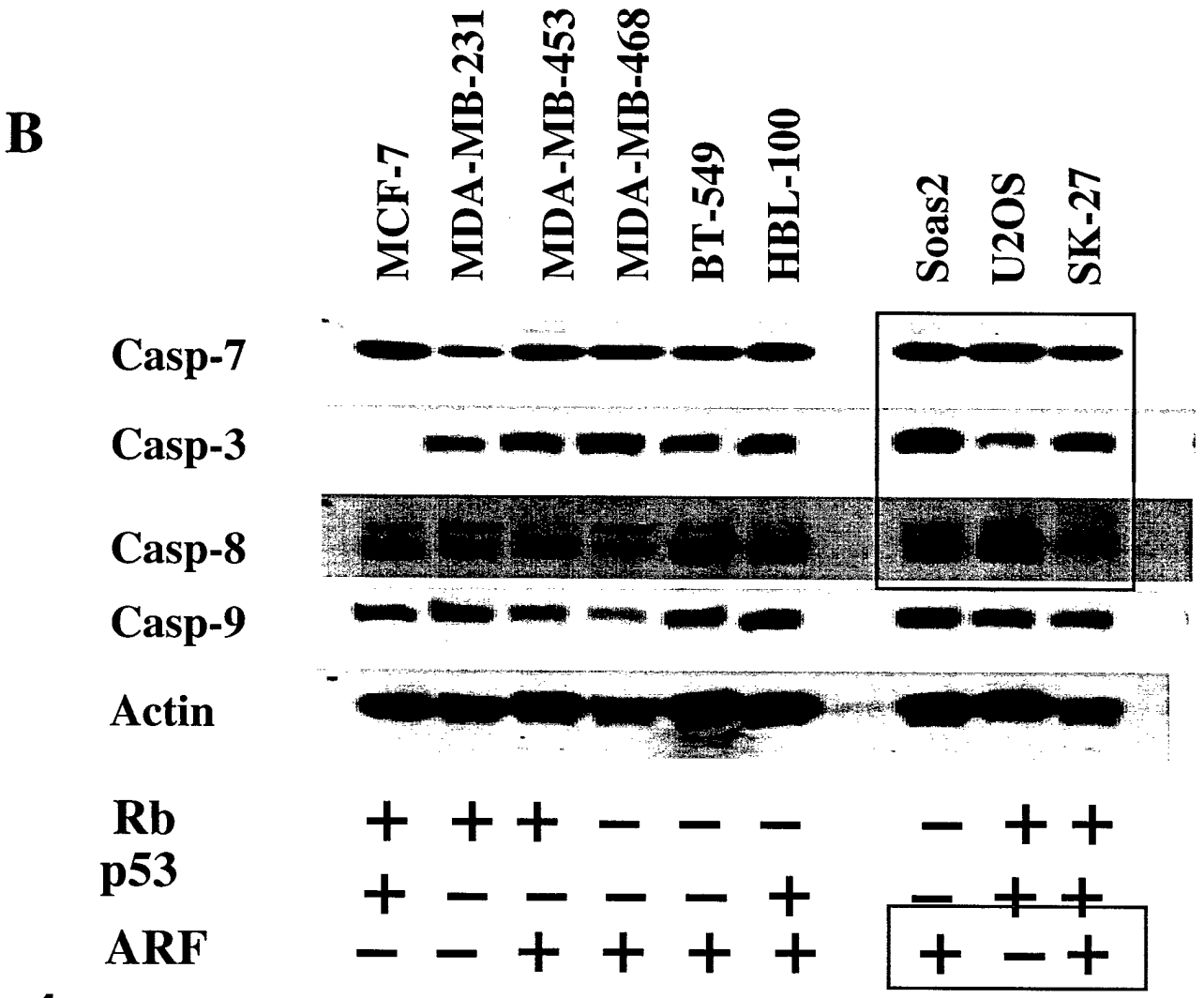


Figure 4

B. Neither p53 nor Rb status affected caspase proenzyme expression in human cancer cells

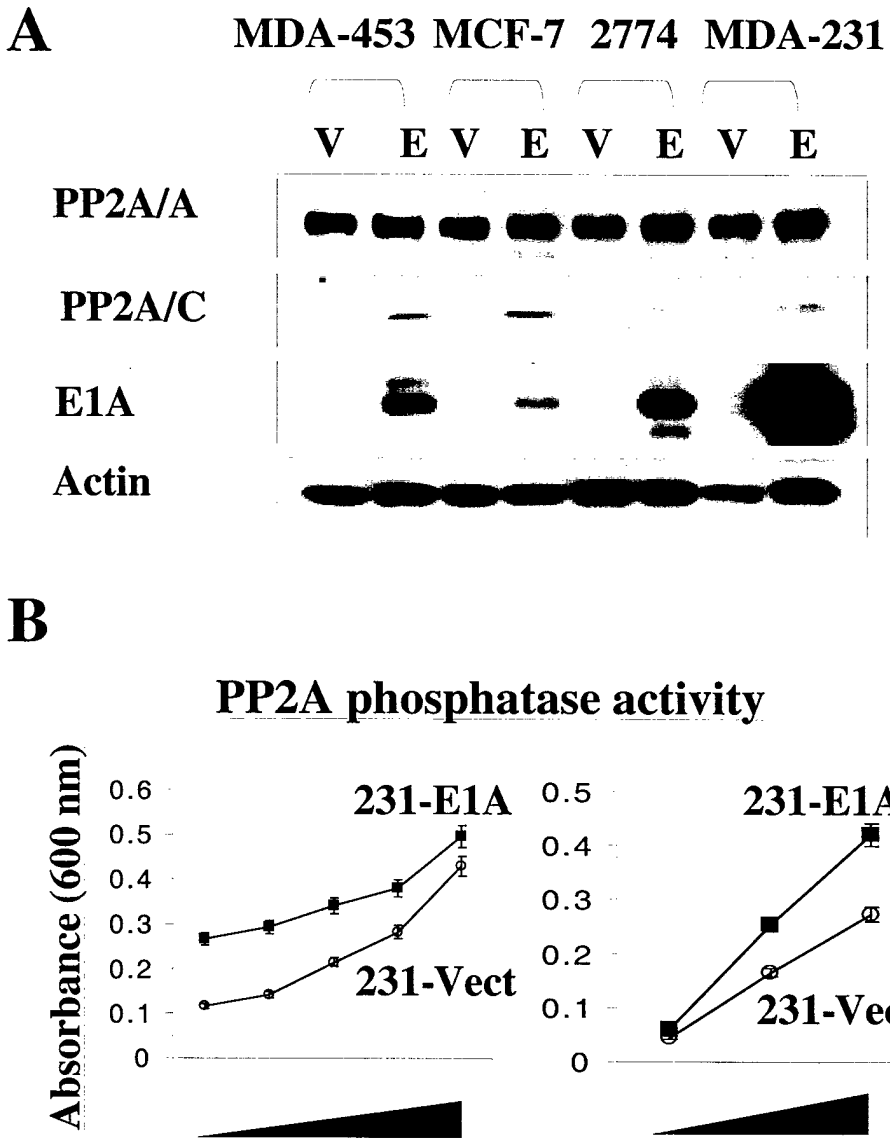


Figure 5.

A. E1A upregulates the catalytic, but not the regulatory subunit of PP2A

B. E1A upregulates PP2A phophatase activity through upregulation of PP2A/C, the catalytic subunit of PP2A.

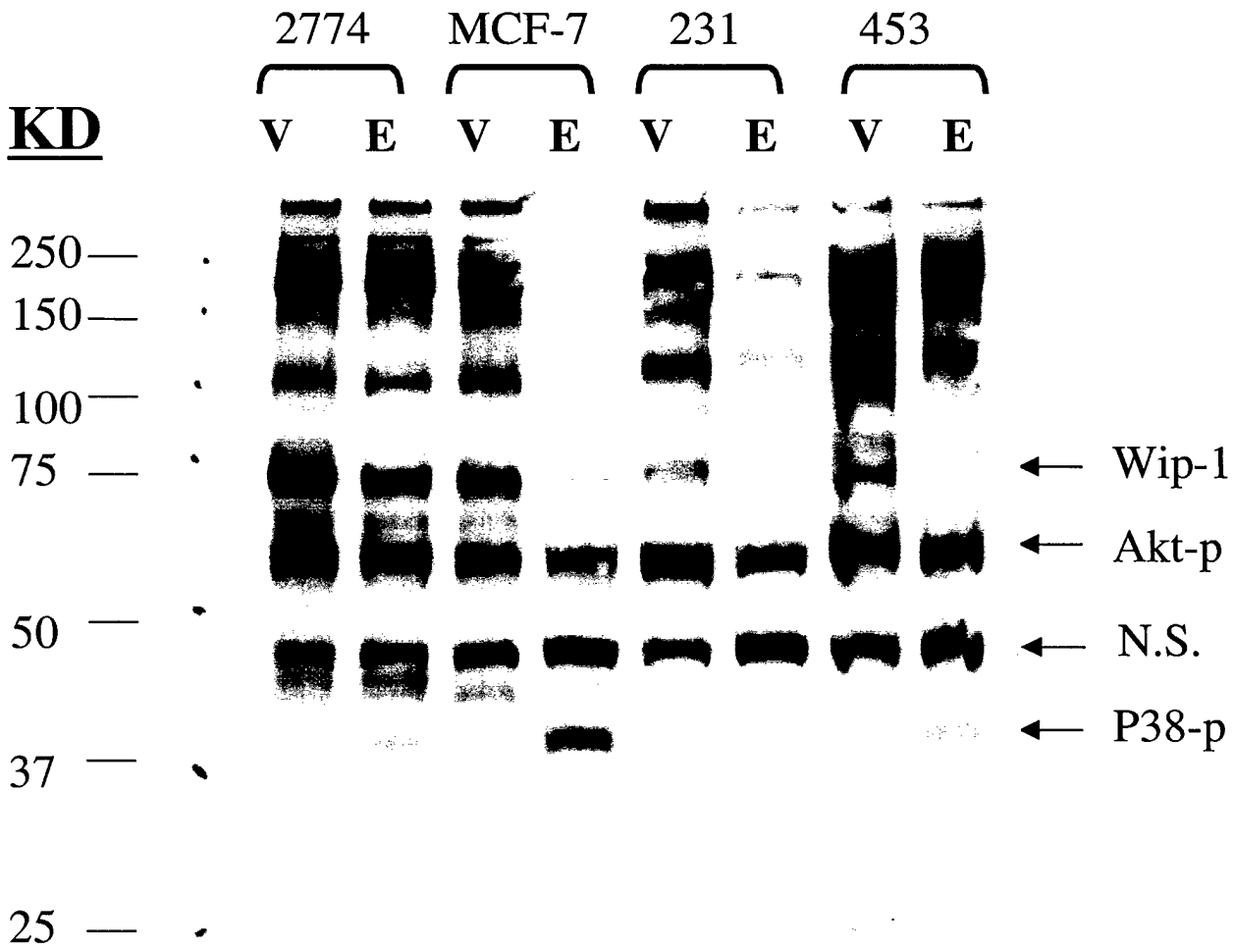
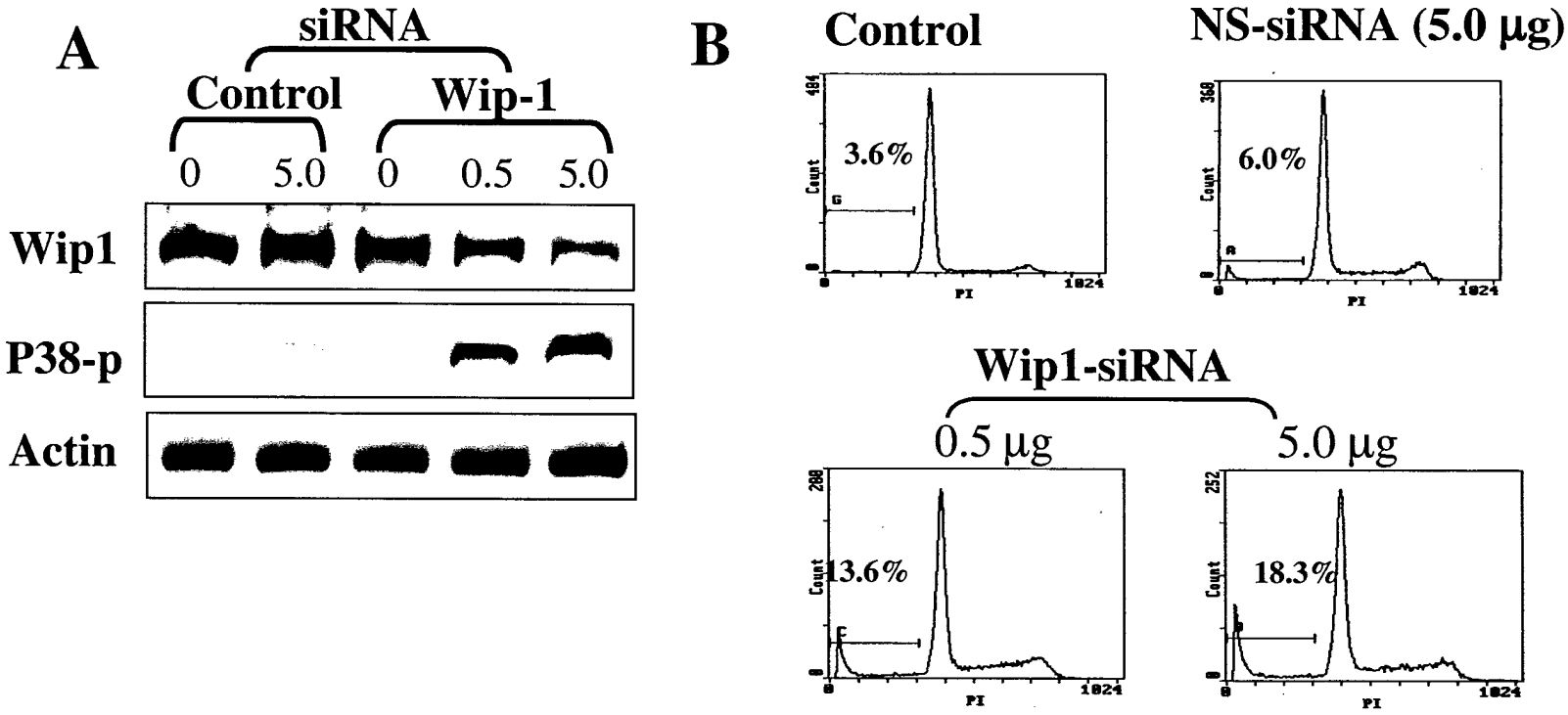


Figure 6 Repression a protein phosphatase PPM1D (Wip-1) expression by E1A in stable E1A expression cells correlates with its effect on Akt and p38 kinase phosphorylation in breast cancer 2774, MCF-7, MDA-MB-231, and MDA-MB-453.

Figure. 7 **Blockage of Wip1 expression by specific siRNA against Wip1 results in enhancement of p38 phosphorylation (A) and spontaneous apoptosis (B) in breast cancer cell line MCF-7 cells.**



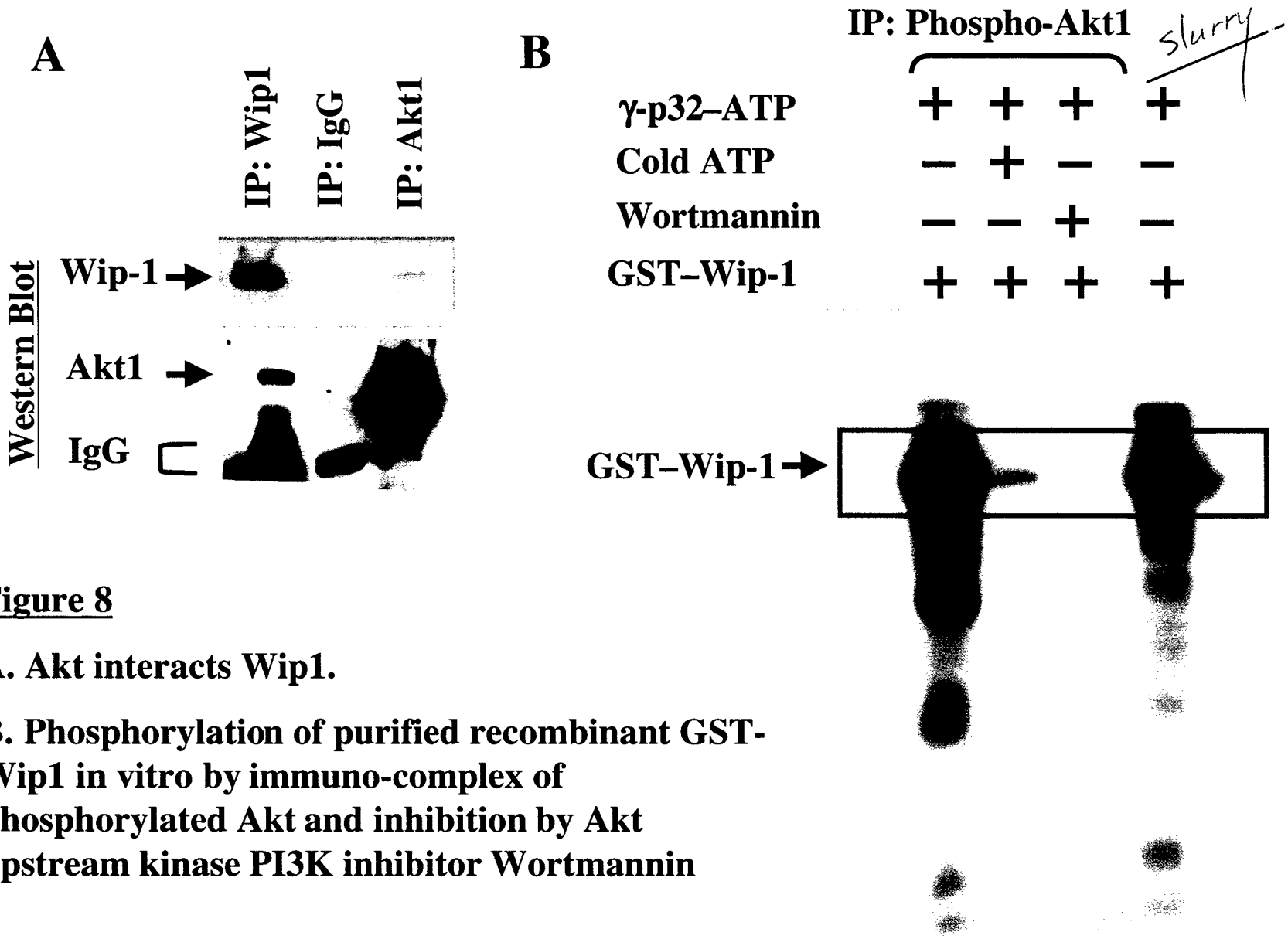


Figure 8

A. Akt interacts Wip1.

B. Phosphorylation of purified recombinant GST-Wip1 in vitro by immuno-complex of phosphorylated Akt and inhibition by Akt upstream kinase PI3K inhibitor Wortmannin

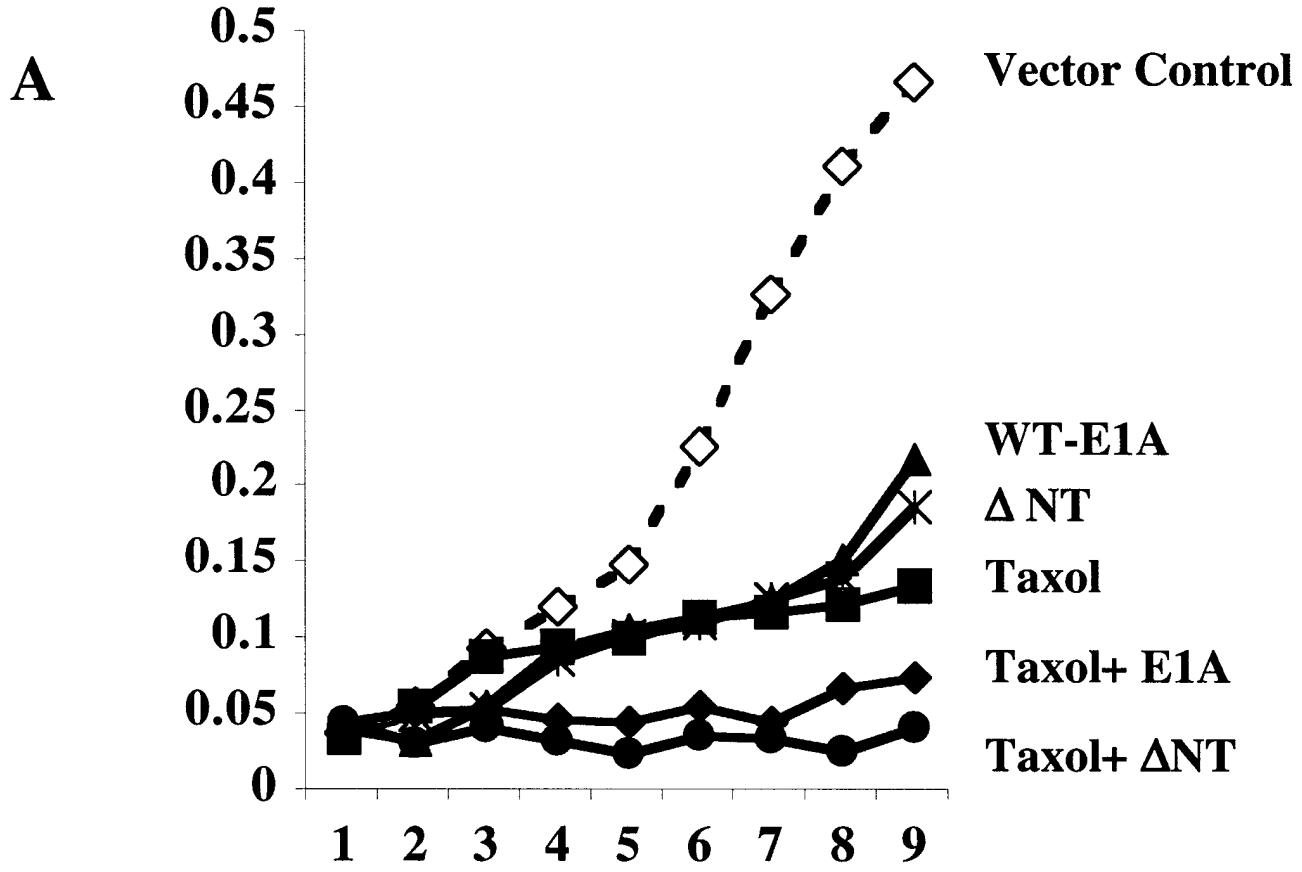


Figure 9.

A. Systemic gene therapy effect of wild-type E1A or N-terminal deletion mutant E1A with or without combination with paclitaxel (Taxol) chemotherapy in breast cancer xenograft established by inoculation with MDA-MB-231 cells in nude mice.

B

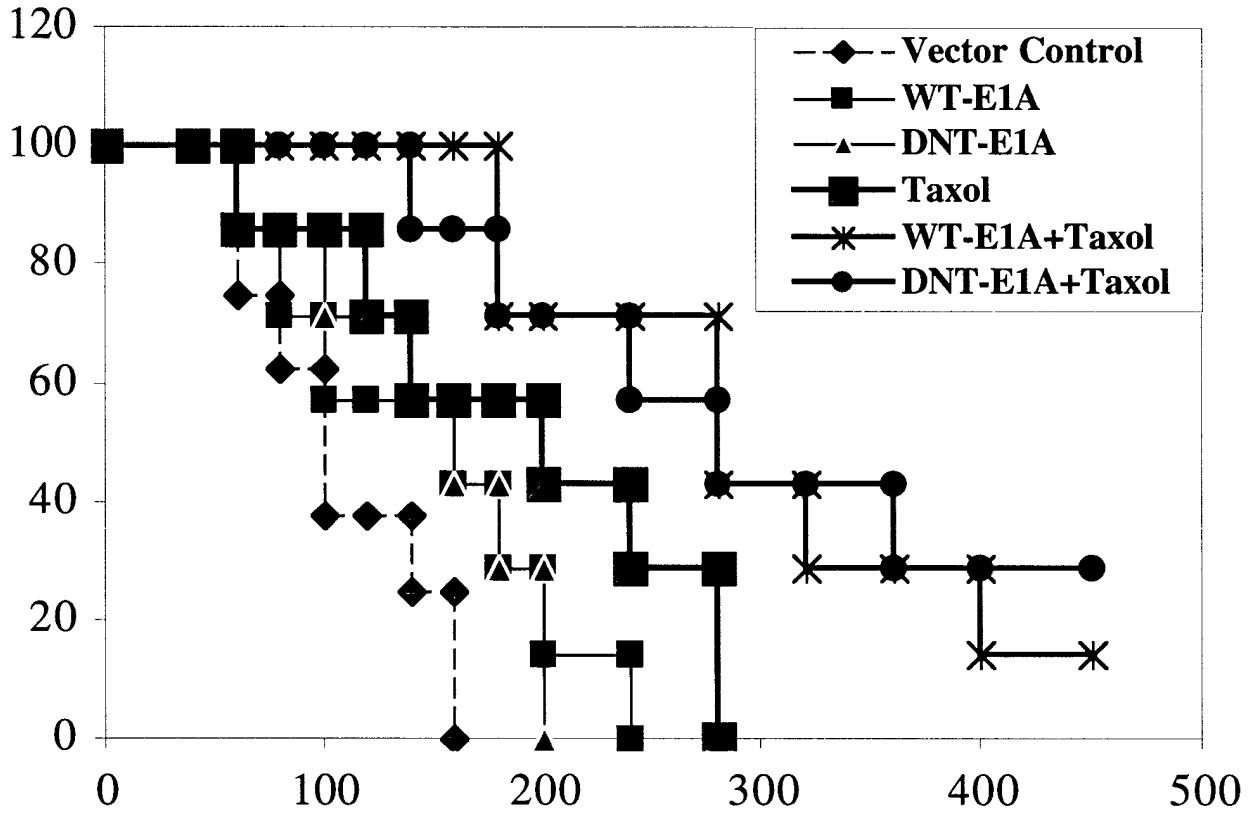


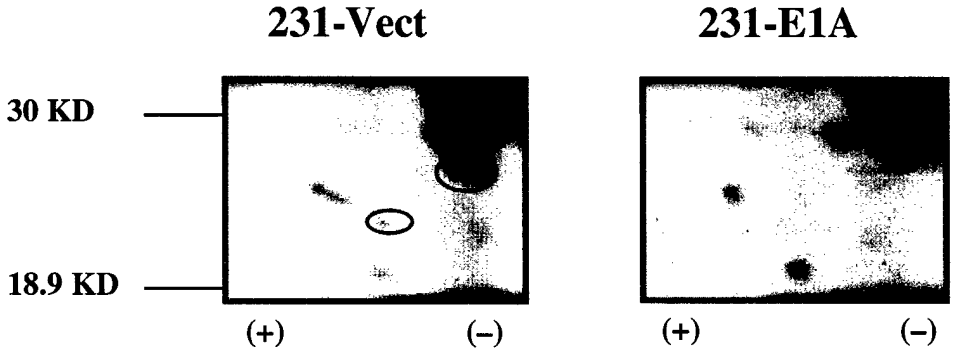
Figure 9.

B. Survival rate of Systemic gene therapy effect of wild-type E1A versus N-terminal deletion mutant E1A with or without combination with paclitaxel (Taxol) chemotherapy in breast cancer xenograft established by inoculation with MDA-MB-231 cells in nude mice.

Figure 10

Proteins differentially expressed in 231-E1A stable cells identified by using 2D gel (A) and Ciphergen protein chip profiling analysis (B)

A. E1A down-regulated proteins identified by 2D gel electrophoresis.



B. Differentially expressed proteins in 231-E1A cells identified by Ciphergen proteinchip analysis

Type of Protein chip	Up-regulated proteins (KD)	Down-regulated proteins (KD)
H4	37.5, 43.7, 48.8, 53.1, 87.4, 4.36, 6.81	45.4, 56.5, 65.7, 69.7, 6.25
SAX2	34.9, 61.1, 7.9	12.4, 17.5, 25.9, 148.8, 6.18
WCX2	37.4, 3.3	69.7
IMAC-cu	37.3, 40.6	49.6, 65.7

MOLECULAR AND CELLULAR BIOLOGY, Oct. 2003, p. 6836–6848 Vol. 23, No. 19
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.19.6836–6848.2003

Regulation of the Activity of p38 Mitogen-Activated Protein Kinase by Akt in Cancer and Adenoviral Protein E1A-Mediated Sensitization to Apoptosis

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Received 19 March 2003/Returned for modification 9 May 2003/Accepted 17 June 2003

The adenoviral early region 1A (E1A) protein mediates sensitization to different stimulus-induced apoptosis, such as tumor necrosis factor alpha, UV and gamma irradiation, and different categories of anticancer drugs. However, the molecular mechanisms underlying E1A-mediated sensitization to apoptosis are still not completely defined. Here, we show that E1A-mediated sensitization to apoptosis by the inactivation of a key survival factor Akt and the activation of a pro-apoptotic factor p38. Also, inactivation of Akt by either a specific inhibitor or a genetic knockout of Akt1 results in p38 activation, possibly through the release of the activity of p38 upstream kinases, including ASK1 and MEKK3. In addition, we showed that p38 phosphorylation is downregulated and Akt phosphorylation is upregulated in multiple human tumor tissues, and this correlates with tumor stage in human breast cancer. A deletion mutation of a conserved domain of E1A, which is required for E1A-induced downregulation of Akt activity, disrupts E1A-mediated upregulation of p38 activity and also eliminates E1A-mediated chemosensitization. Thus, activation of p38 and inactivation of Akt may have general implications for tumor suppression and sensitization to apoptosis.

Many types of tumors are associated with activated oncogenic kinases, and two complementary roles of these oncogenic kinases are stimulating signaling pathways that enable cells to function independent of their environment and making tumor cells resistant to genotoxic therapies, such as chemo- and radiotherapy (22, 24, 48). Deregulated growth signaling pathways and acquired resistance toward apoptosis therefore constitute two hallmarks of most, if not all, human tumors (18). For example, it has been shown that the serine/threonine kinase Akt and its family members Akt 2 and 3 are either amplified or their activity is constitutively elevated in human carcinomas such as breast, pancreatic, ovarian, brain, prostate, and gastric adenocarcinomas (39, 50). As it is a direct downstream target of phosphatidylinositol 3-kinase (PI3K), Akt is also a key oncogenic survival factor and can phosphorylate and inactivate a panel of critical proapoptotic molecules, including Bad, caspase 9, the Forkhead transcription factor FKHRL1 (known to induce expression of proapoptotic factors such as Fas ligand), GSK3-β, cell cycle inhibitors p21 and p27, and tumor suppressor TSC2, etc. (4, 25, 39, 50, 58). Akt can also inactivate p53, a key tumor suppressor, through phosphorylation and nuclear localization of MDM2 (33, 50, 59). Activation of Akt has been shown to induce resistance to apoptosis induced by a range of drugs (41). Thus, molecules that can block Akt activity may have important significance in cancer therapy and drug sensitization.

The adenovirus early region 1A protein (E1A) induces chemosensitization among different categories of anticancer drugs, including cisplatin, adriamycin, etopside, staurosporine, 5-fluorouracil, and paclitaxel (Taxol) (5, 14, 16, 32, 45, 53), suggesting that a general cellular mechanism may exist to regulate E1A-mediated chemosensitization. However, the molecular mechanisms underlying E1A-mediated chemosensitization are still not completely defined. Earlier studies on normal fibroblast cells revealed that E1A-mediated sensitization to cytotoxic anticancer drugs depends on the expression of functional p53 and p19ARF, an alternative splicing form of p16INK4a (12, 31, 32). E1A was also shown to downregulate Her-2/neu overexpression and facilitate E1A-mediated sensitization to the cytotoxicity of anticancer drugs in human breast and ovarian cancer cells (53, 55, 56). In another study, E1A was reported to mediate sensitization to anticancer drugs in human osteosarcoma cells (16) in a p53- and Her-2/neu-independent manner. Similarly, there is no correlation between p53 protein level and sensitivity of DNA-damaging agents in keratinocytes carrying adenovirus E1A (45). A few other critical molecules were also proposed to be involved in E1A-induced chemosensitization, such as the proapoptotic protein Bax, caspase 9, or a yet unidentified inhibitor that ordinarily provides protection against cell death (14, 15, 34, 43, 49, 52). However, none of the above molecules or pathways can really serve as a general cellular mechanism for E1A-mediated sensitization to apoptosis in a diverse cellular context. Recently, transcriptional upregulation of procaspases (such as pro-caspase 3, 7, 8, and 9) through E1A-mediated disruption of pRB function and subsequent release of free E2F-1 was reported to contribute to both p53-dependent and p53-independent drug sensitization by E1A in diploid normal fibroblast cells (37). In the present study, we found that E1A can activate p38 and inactivate Akt and showed that this pathway may provide a general cellular mechanism for E1A to mediate sensitization to different categories of anticancer drugs.

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MATERIALS AND METHODS

Cell culture, cell harvest, and Western blot. Human breast, ovarian, prostate, pancreatic, and colon cancer cell lines were grown in Dulbecco's modified Eagle's medium–F-12 (Life Technologies, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines in breast cancer MDA-MB-231 and MCF-7 were established as described previously (36, 52). Similarly, domain deletion mutant constructs of E1A were transfected into MDA-MB-231 cells, and stable clones were screened and selected in the presence of G418. Akt1 knockout mouse embryonic fibroblasts (MEFs) and myristoylated, membrane-bound, constitutively active Akt1 (myr-Akt)-transfected stable Rat1 cells were provided by Nissim Hay (University of Illinois at Chicago, Chicago) (10).

For the analysis of basal Akt and p38 expression and activity, cells were serum starved overnight before harvesting. Cells were then washed twice with cold phosphate-buffered saline (PBS) and lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate ($NaVO_3$), and 1.5% aprotinin. The cell extracts were clarified by centrifugation, and protein concentrations were determined by using a Bio-Rad (Hercules, Calif.) protein assay reagent and analyzed in a spectrophotometer with bovine serum album (Sigma, St. Louis, Mo.) as the protein standard. Aliquots of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore Corp., Bedford, Mass.) by using standard procedures. The membranes were then subjected to Western blotting, and the blots were developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, N.J.).

Primary antibodies. For Western blot analysis, rabbit polyclonal antibodies against phospho-Akt (Ser 473, catalog no. 9271, 1:1,000 dilution) and phospho-p38 (Thr 180/Tyr 182, catalog no. 9211, 1:1,000 dilution) were purchased from Cell Signaling Technology (Beverly, Mass.). Rabbit polyclonal antibodies against nonphosphorylated total Akt (catalog no. 9272, 1:500 dilution), p38 (catalog no. 9212, 1:500 dilution), and cleaved poly(ADP-ribose) polymerase (PARP) (Asp214, catalog no. 9541, 1:1,000 dilution) were also from Cell Signaling Technology. A rabbit polyclonal antibody against beta-actin was used as a loading control for Western blotting and was purchased from Sigma. For immunohistochemical (IHC) study, we used an IHC-specific rabbit polyclonal antibody against phospho-Akt (Ser 473, catalog no. 9277) or an IHC-specific monoclonal antibody against phospho-p38 (Thr180/Tyr 182, catalog no. 9216) at a 1:75 dilution for both antibodies. The monoclonal antibody used against the E1A proteins was M58 (PharMingen, San Diego, Calif.). Rabbit polyclonal anti-Bax antibodies, a hamster anti-human Bcl-2 monoclonal antibody, and rabbit polyclonal anti-ASK1 (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Mouse monoclonal antibodies against human caspase 3 and 7 were from Transduction Laboratories (1:1,000 dilution, C31720; Lexington, Ky.) and BD PharMingen (1:1,000 dilution, 66871A), respectively. Rabbit polyclonal antibodies against human caspase 8 and 9 were from Santa Cruz Biotechnology (1:500 dilution, SC-7890/H-134) and Cell Signaling Technology (1:500 dilution, no. 9502), respectively. To detect hemagglutinin (HA)-tagged proteins, a monoclonal anti-HA antibody was used (1:1,000 dilution, catalog no. 1,583,816; Boehringer Mannheim, Indianapolis, Ind.). A monoclonal anti-FLAG antibody (M2) was purchased from Sigma (1:1,000 dilution).

Transient transfection, MTT assay, luciferase assay, and FACS analysis. The standard MTT assay was performed to measure the viable cells after treatment with anticancer drugs as described previously (52). Expression vectors for HA-p38, constitutively active Akt (CA-Akt), dominant-negative Akt (DN-Akt), and cytomegalovirus driving luciferase (pcDNA3-Luc) were used in this study. First, 10^5 cells in a 60-mm dish were transfected with 2.2 μg of total DNA by using the DC-Chol cationic liposome as described previously (52). After 48 h, the cells were split into three sets: one used for a luciferase assay after exposure with or without paclitaxel for 24 h, one used to analyze Akt and p38 protein expression, and one fixed in 75% ethanol, stained with propidium iodide (25 μg/ml), and sent for fluorescence-activated cell sorter (FACS) analysis. The percentage of paclitaxel-treated cells that exhibited luciferase activity was normalized by using the luciferase activity of the untreated cells as the baseline (100%). Standard deviations from three independent experiments were calculated.

Establishment of IPTG-inducible DN-p38 stable cell lines. One E1A-expressing MDA-MB-231 clone was cotransfected with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible DN-p38α construct (a gift from Philipp E. Schere, Albert Einstein College of Medicine, Bronx, N.Y.) and the plasmid pCMVLacI (Stratagene, La Jolla, Calif.). Stable clones were selected in the presence of 200 μg of hygromycin/ml.

Immunoprecipitation. After transient transfection with HA-tagged p38 or CA-Akt, cells were stimulated with 10 μM insulin for 15 min. Cells were then lysed, and cell lysates were centrifuged at 16,000 *g* for 30 min. The supernatants were then transferred to a fresh tube. Proteins were cleared via addition of a normal mouse or rabbit immunoglobulin G and immunoprecipitated with anti-p38, anti-Akt, or anti-HA antibodies. Immunoprecipitates were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Akt, p38, and ASK1 were detected by Western blotting.

Kinase assay. Nonradioactive kinase assay kits for p38 and Akt were purchased from Cell Signaling (New England BioLabs, Beverly, Mass.). The p38 and Akt kinase activities were measured according to the manufacturer's protocol with glutathione *S*-transferase (GST)–ATF-2 as the substrate for p38 and GST–GSK-3-β as the substrate for Akt.

Tissue microarray and immunohistochemistry. Tissue microarray slides (HistoArray no. IMH-343/BA2 and IMH-304/CB2) were purchased from IMGENEX (San Diego, Calif.). Detailed information about each slide is available online. Slide processing and immunohistochemical staining were performed according to the manufacturer's protocol. Briefly, tissue slides were heated at 60°C, deparaffinized in xylene, hydrated in graded ethanol, and then immersed in tap water. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 for 20 min in a 95°C water bath. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide solution followed by three sequential PBS washes (5 min each). Slides were then blocked by the respective normal serum for each primary antibody, incubated with primary antibody diluted in TBS-T (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween 20) containing 1% ovalbumin and 1 mg of sodium azide/ml, incubated with biotinylated secondary antibody for 30 min at room temperature, washed with PBS again, and incubated with avidin-biotin complex (Vestastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, Calif.). Slides were washed with PBS again, incubated with the AEC (3-amino-9-ethylcarbazole) substrate kit (catalog no. Sk-4200; Vector Laboratories, Inc.), and then counterstained in Meyer's xylene.

One representative slide per case was evaluated with the antibodies mentioned above. The intensities of staining seen in different areas of the same slide were analyzed according to criteria described previously in the literature (1). The intensity was designated 0 when no cells stained, 1+ when 10 to 20% of cells stained (weak), 2+ when 20 to 50% of cells stained (moderate), and 3+ when more than 50% of cells stained (strong).

Statistics. For statistical analysis, groups scored as 0 and 1+ were combined as weak staining while groups scored as 2+ and 3+ were combined as strong staining. Similarly, to simplify the statistical analysis, breast tumors with stages 1 and 2 were combined as early stages of tumors ($n = 25$ cases) while tumors with stages 3 and 4 were combined as late stages of tumors ($n = 25$ cases). Statistical analysis was performed by using χ^2 analysis.

RESULTS

E1A upregulates p38 activity and downregulates Akt activity. To determine whether apoptosis-related kinases are involved in E1A-mediated sensitization to apoptosis, we examined the phosphorylation status of three well-known kinases involved in regulation of apoptosis, p38, Akt, and JNK, in E1A-expressing MDA-MB-231 and MCF-7 cells (231-E1A and MCF-7-E1A) versus vector-transfected cells (231-Vect and MCF-7-Vect). We detected phosphorylated p38 in cells stably expressing E1A but not in vector-transfected cells. However, the level of phosphorylated Akt was much higher in vector-transfected cells than in E1A-expressing cells. The levels of total Akt and p38 were similar in both types of cells (Fig. 1A). Kinase assays showed that p38 activity was higher and Akt activity was lower in 231-E1A and MCF-7-E1A cells than in 231-Vect and MCF-7-Vect cells (Fig. 1B and C). We did not detect any difference in the level of phosphorylated JNK between E1A-expressing and vector-transfected cells (data not shown). These results indicated that E1A enhanced the activity of the proapoptotic kinase p38 and repressed the activity of the antiapoptotic kinase Akt but did not affect JNK phosphorylation.

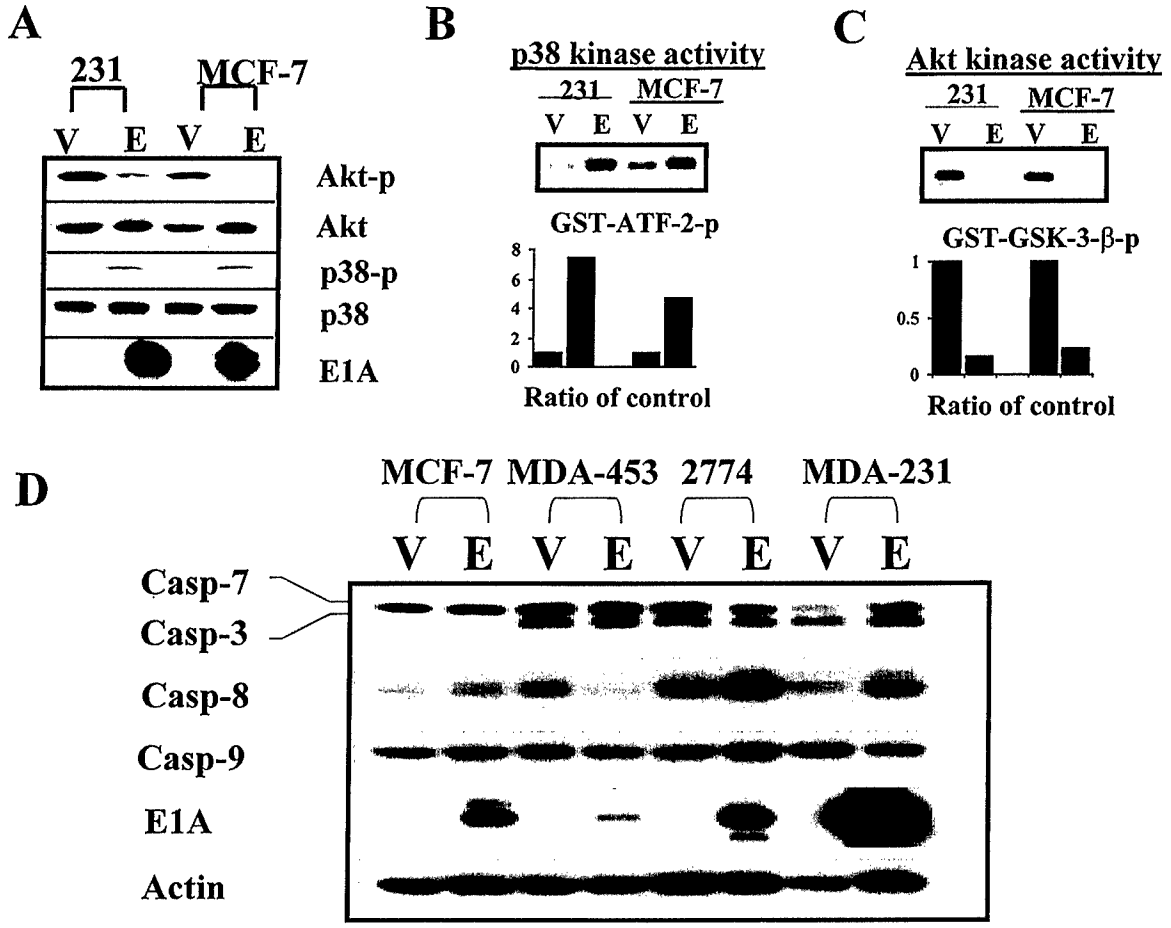


FIG. 1. Upregulation of p38 activity and downregulation of Akt activity by E1A correlated with E1A-mediated sensitization to paclitaxel-induced apoptosis. (A) Phospho-p38 (p38-p) and phospho-Akt (Akt-p) levels in E1A-expressing cells versus those in vector-transfected MDA-MB-231 and MCF-7 cells are shown. Total p38 (p38) and Akt (Akt) were used as loading controls. Results of kinase assays of p38 (B) and Akt (C) and densitometric analysis of relative p38 activity with GST–ATF-2 as a substrate and Akt activity with GST–GSK-3-β as a substrate in E1A-expressing cells versus vector-transfected control cells. E, E1A-expressing cells; V, vector transfected control cells. (D) Expression of caspase 3, 7, 8, and 9 proenzymes in E1A stable cells established in human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-453 and ovarian cancer cell line 2774. V, vector control; E, E1A stable cells.

A recent report showed that transient transfection of E1A resulted in the accumulation of caspase proenzymes in human normal diploid fibroblasts (37). We therefore compared the expression levels of caspase proenzymes caspase 3, 7, 8, and 9 between E1A transfectants and vector-transfected carcinoma cells, including breast cancer MDA-MB-231, MDA-MB-453, and MCF-7 cells and ovarian cancer 2774 cells. Unlike what was demonstrated with normal fibroblast cells, we did not observe a unanimous increase of these caspase proenzymes in the E1A stable cells established in human cancer cells with epithelial origins (Fig. 1D). This suggests that transcriptional upregulation of the caspase proenzymes in these human cancer cells may not be as critical as it is in the normal fibroblast cells and that other cellular mechanisms may exist for the E1A-mediated sensitization to apoptosis in human cancer cells.

Upregulation of p38 activity and downregulation of Akt activity correlate with E1A-mediated sensitization to paclitaxel-induced apoptosis. To test whether alteration of the kinase activity of Akt or p38 played a role in E1A-mediated sensitization to apoptosis, we compared the kinetics of phosphorylation of Akt or p38 with paclitaxel-induced apoptosis in 231-E1A cells by using PARP cleavage and Bcl-2 phosphorylation as apoptotic cell death markers. PARP cleavage and Bcl-2 phosphorylation occurred after decreased Akt phosphorylation and increased p38 phosphorylation in 231-E1A cells after exposure to 0.01 μM paclitaxel (Fig. 2A). However, no significant change was detected in the protein levels of p53 and Bax (Fig. 2A). The same concentration of paclitaxel did not trigger PARP cleavage, induce Bcl-2 phosphorylation, or modulate the levels of phosphorylated p38 and Akt in the parental MDA-MB-231 cells (data not shown). To trigger a similar response in parental MDA-MB-231 cells, a much higher dosage was required (Fig. 2B). The results suggest that downregulation of Akt and upregulation of p38 activities may be involved in the E1A-mediated sensitization to paclitaxel-induced apoptosis.

Activation of p38 and inactivation of Akt are required for E1A-mediated sensitization to drug-induced apoptosis. To

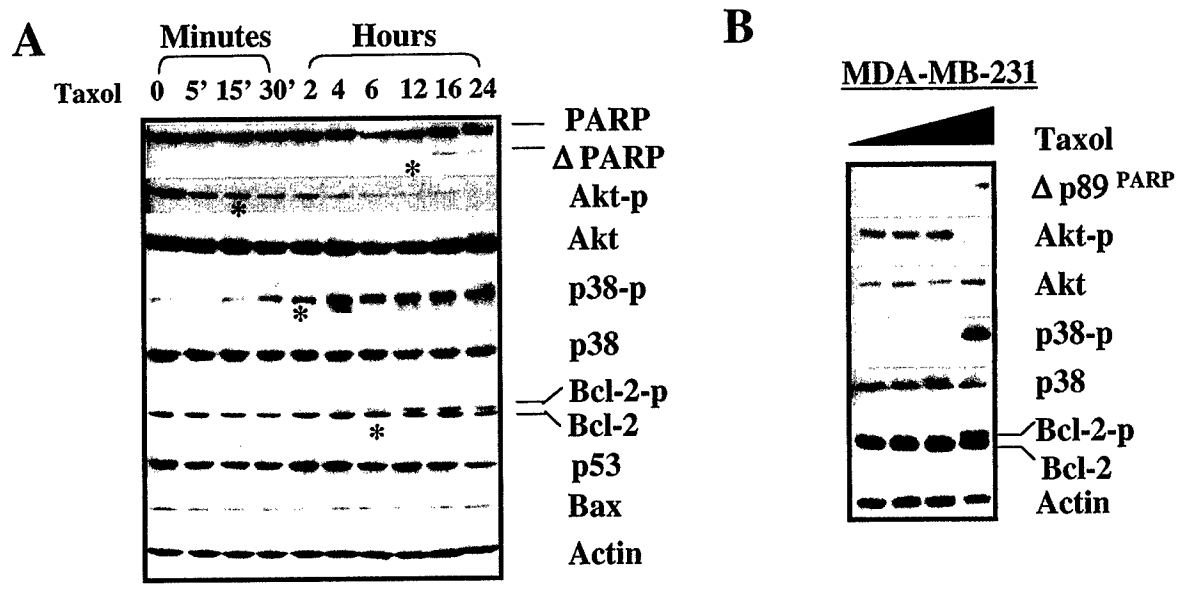


FIG. 2. Upregulated p38 activity and downregulated Akt activity correlate with E1A-mediated sensitization to paclitaxel-induced apoptosis. (A) Kinetics of PARP, Akt, p38, Bcl-2, p53, and Bax protein expression in 231-E1A cells before and after exposure to 0.01 μM paclitaxel. Bcl-2 phosphorylation and PARP cleavage were detected at 6 and 12 h after exposure to paclitaxel and became more obvious thereafter. Phosphorylation of p38 was first detected after 30 min to 2 h and then become more obvious after 4 h of treatment. Dephosphorylation of Akt could be detected even earlier, at 5 min posttreatment. Actin was used as a loading control. A dramatic alteration of each molecule was marked with an asterisk. (B) Dose-dependent effect of PARP cleavage and Akt and p38 phosphorylation in MDA-MB-231 cells after exposure to paclitaxel for 24 h. The concentrations of paclitaxel used ranged from 0 to 0.001, 0.01, and 0.1 μM.

evaluate whether activation of p38 is required for E1A-mediated sensitization to paclitaxel, we tested whether blocking p38 activity could inhibit E1A-mediated sensitization in 231-E1A cells. We used the specific p38 inhibitor SB203580 (Fig. 3A) and a DN-p38 mutant to block p38 activation (Fig. 3B). A pcDNA3-Luciferase (pcDNA-Luc) construct was transfected into 231-E1A cells, and luciferase activity was used as a measurement for cell survival. Pretreatment with SB203580 inhibited the phosphorylation of p38 in cells with or without exposure to paclitaxel (Fig. 3C, lanes 3 to 4) and protected cells from a paclitaxel-induced decrease of luciferase activity (Fig. 3C, lane 2 versus lane 4). In addition, FACS analysis showed that pretreatment with SB203580 protected 231-E1A cells from paclitaxel-induced apoptosis (27.5% versus 18.0%) (Fig. 3D, lanes 2 and 4, bottom). These data suggest that p38 activation is required for E1A-mediated sensitization to paclitaxel-induced apoptosis. Using a DN-p38 to block p38 activation further supported the above results (Fig. 3B). When the cells were switched to medium containing 5 μM IPTG for 24 h, expression of IPTG-inducible DN-p38 was induced in the presence or absence of paclitaxel (Fig. 3B, lower panel). FACS analysis showed that induction of DN-p38 by IPTG significantly inhibited paclitaxel-induced apoptosis (Fig. 3B, upper panel). Identical results were obtained when two additional stable clones were studied (data not shown). However, IPTG could not induce this effect in the 231-E1A cells without IPTG-induced DN-p38 (data not shown). Taken together, these data suggest that p38 activation is required for E1A-mediated sensitization to paclitaxel-induced apoptosis.

To determine whether downregulation of Akt activity is also required for E1A-mediated sensitization to paclitaxel, we examined whether activation of Akt by transfection of CA-Akt would inhibit paclitaxel-induced apoptosis in 231-E1A cells. The level of phosphorylated Akt and luciferase activity was increased in CA-Akt-transfected 231-E1A cells compared with that in the control 231-E1A cells (Fig. 3C, lanes 1 and 2 versus lanes 5 and 6). FACS analysis showed that fewer apoptotic cells were detected in CA-Akt-transfected cells (15.9%) than in control 231-E1A cells (27.5%) after exposure to paclitaxel (Fig. 3D, lane 2 versus lane 6, bottom). Thus, inhibition of Akt phosphorylation is also required for E1A-mediated sensitization to paclitaxel-induced apoptosis.

Activation of p38 and inactivation of Akt represent a general cellular mechanism in response to different apoptotic stimuli. To determine whether the same mechanism of E1A-mediated sensitization to paclitaxel applies to other anticancer drugs, we tested the effects of four additional drugs used in the clinic for treatment of human cancer. These four anticancer drugs induce antitumor activities through different modes of action: doxorubicin-adriamycin (topoisomerase II inhibitor), cisplatin (DNA-damaging agent), methotrexate (antimetabolite drug), and gemcitabine (antimetabolite drug). The expression of E1A significantly enhanced each drug's cytotoxicity in MDA-MB-231 cells, determined by using the MTT assay (Fig. 4A). In addition, downregulation of Akt phosphorylation and upregulation of p38 phosphorylation and PARP cleavage were observed in 231-E1A cells but not in 231-Vect cells treated with each of the drugs at the same dosage (Fig. 4B). These results suggest that activation of p38 and inactivation of Akt may contribute to E1A-mediated sensitization to apoptosis induced by these different drugs.

To address whether activation of p38 and inactivation of Akt

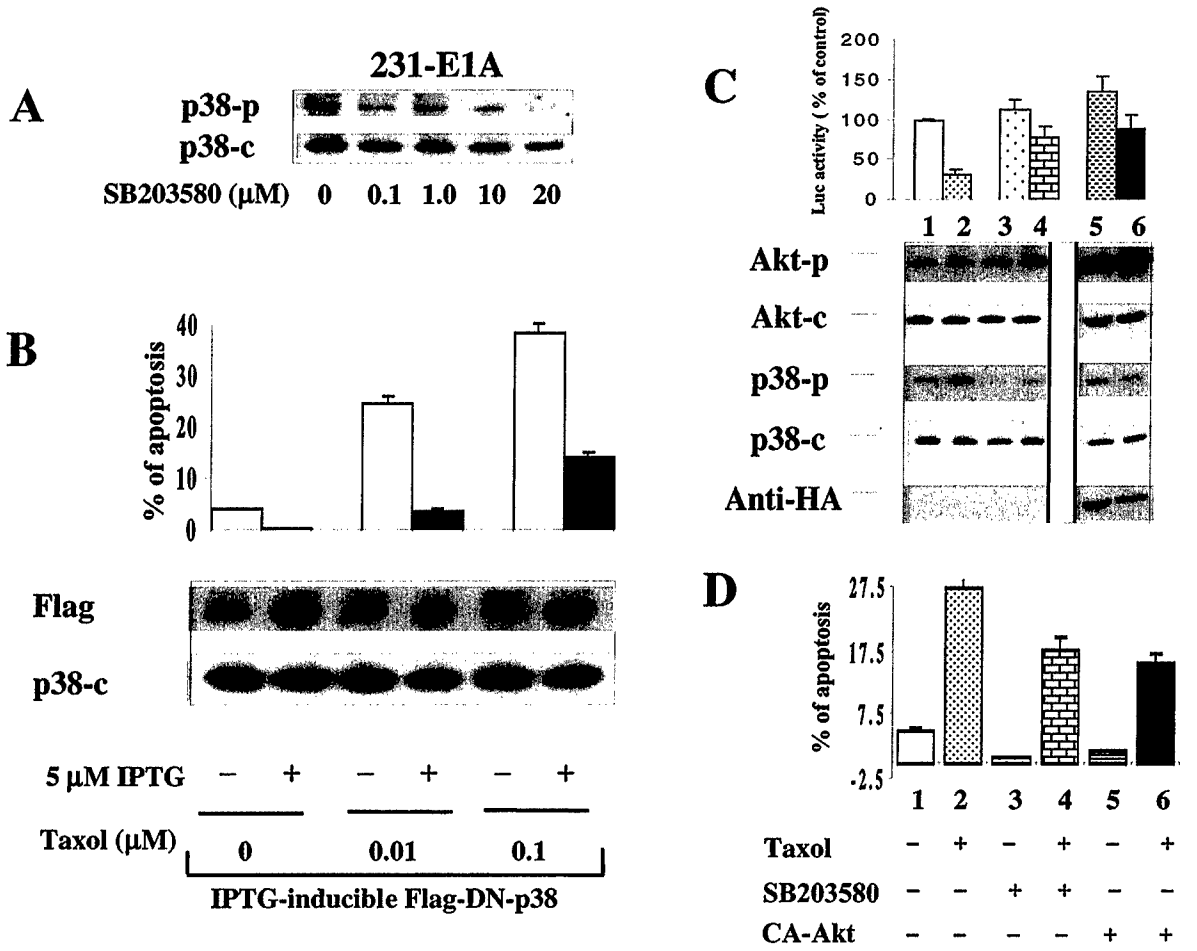


FIG. 3. Activation of p38 and inactivation of Akt are required for E1A-mediated sensitization to paclitaxel-induced apoptosis. (A) Dose-dependent effect of SB203580 on p38 phosphorylation in 231-E1A cells. (B) An IPTG-inducible, Flag-tagged DN-p38 stable cell clone was established in 231-E1A cells. Repression of p38 activity by IPTG-inducible DN-p38 enhances Akt phosphorylation and eliminates E1A-mediated sensitization to paclitaxel in E1A-expressing cells in the presence of 5 μM IPTG for 24 h. (C) Western blot analysis of p38 and Akt in E1A-expressing MDA-MB-231 cells and luciferase assay. The viability of cells with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) exposure to paclitaxel was measured by luciferase assay. The pcDNA3-Luc vector was cotransfected into 231-E1A cells with (lanes 5 and 6) or without a HA-tagged, myristoylated, membrane-targeted CA-Akt (lanes 1 to 4) before treatment with paclitaxel. After exposure to paclitaxel for 4 h, a portion of cells was harvested for protein extraction while the rest were grown for 24 h. In the absence of 20 μM SB203580, the level of phosphorylated p38 increased after exposure to paclitaxel (lanes 1 and 2). Expression of CA-Akt was detected with an anti-HA monoclonal antibody (lanes 5 and 6). (D) A portion of the above-described cells was also subjected to FACS analysis to measure apoptosis. +, present; −, absent.

also applied to drug-induced apoptosis in the absence of E1A, we tested whether increasing the dosage of gemcitabine or adriamycin could also enhance p38 activation and inhibit Akt activation, which would then contribute to drug-induced apoptosis. When MDA-MB-231 and HBL-100 cells were exposed to a dose of gemcitabine or adriamycin 10 times higher than that used in experiment whose results are shown in Fig. 4B, we observed a similar pattern of downregulation of Akt and upregulation of p38 activation, which was correlated with PARP cleavage (Fig. 4C). Similar results were also observed in MDA-MB-231 cells when exposed to a higher dose of paclitaxel (Fig. 2B). These results suggest that p38 activation and Akt inactivation may not be limited to E1A-mediated sensitization to apoptosis but may also contribute to drug-induced apoptosis in the absence of E1A. Thus, downregulating Akt activity and upregulating p38 activity may represent a general cellular mechanism of response to apoptotic stimuli, and E1A may turn on this cellular mechanism and mediate sensitization to drug-induced apoptosis.

To determine the physiological relevance of inactivation of Akt and activation of p38 in the execution of apoptosis, we extended our investigation to apoptosis induced by serum starvation, tumor necrosis factor alpha (TNF-α), and UV irradiation. We observed that phosphorylation of p38 and dephosphorylation of Akt were correlated with serum starvation-, TNF-α-, and UV-induced PARP cleavage in 231-E1A cells, especially in detached apoptotic cells (Fig. 4D, lanes 1 to 8). However, a dose of 10 times higher is required for inducing a response in parental MDA-MB-231 cells similar to that in 231-E1A cells (Fig. 4D, lanes 9 to 11). Taken together, down-

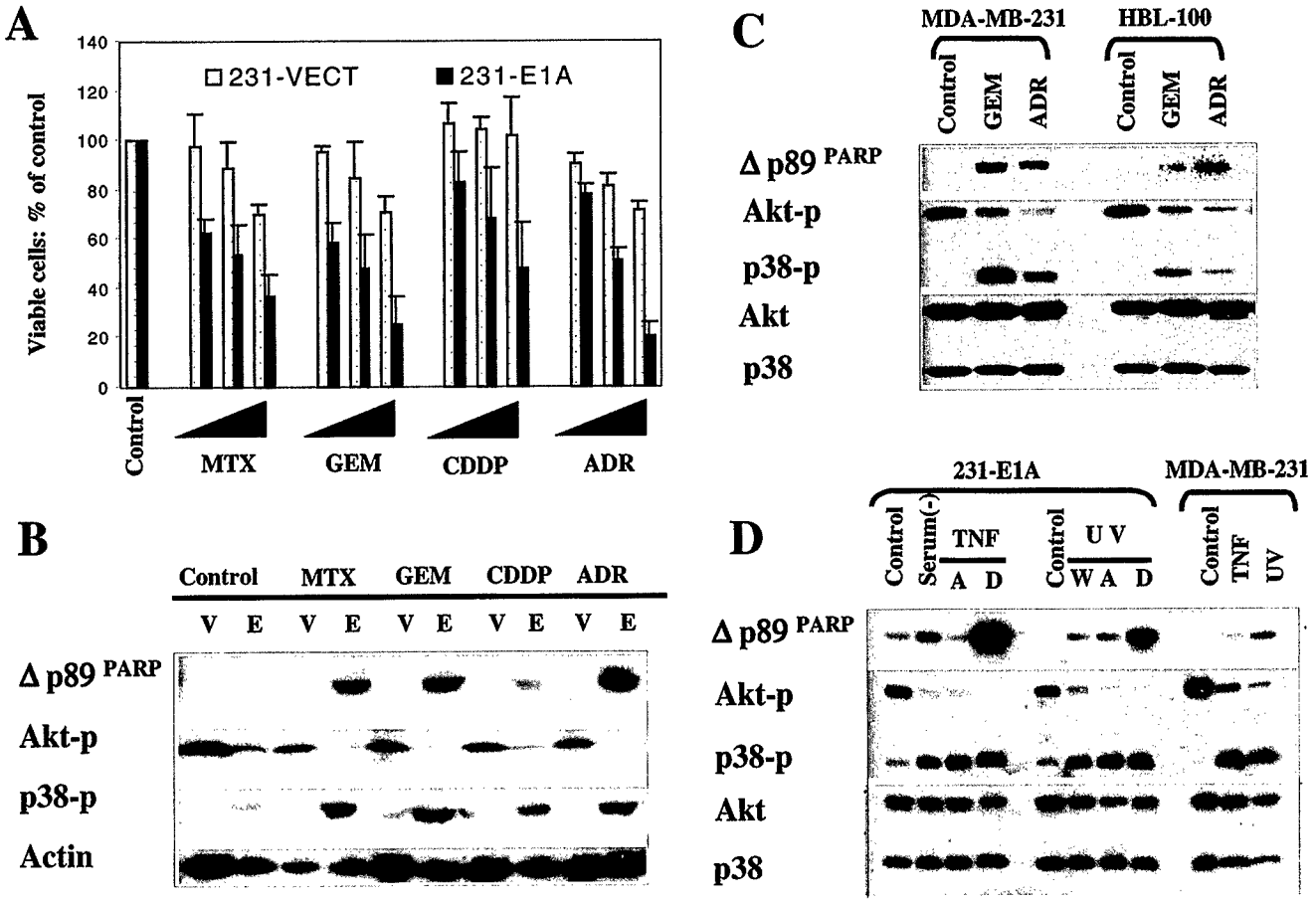


FIG. 4. Activation of p38 and inactivation of Akt represent a general cellular mechanism in response to different apoptotic stimuli. (A) Percentage of viable cells in vector-transfected (231-Vect) and E1A-expressing MDA-MB-231 (231-E1A) cells after exposure to different doses of adriamycin (ADR) (0.1, 1.0, and 10 μM), cisplatin (CDDP) (0.2, 2, and 10 μg/ml), gemcitabine (GEM) (0.2, 2, and 10 μg/ml), and methotrexate (MTX) (0.2, 2, and 10 μM) for 24 h. Cell viability was measured by using the MTT assay. (B) Downregulation of Akt activation and upregulation of p38 activation correlated with drug-induced PARP cleavage in 231-Vect (V) and 231-E1A (E) cells. The concentrations used were 1 μM ADR, 2-μg/ml CDDP, 2-μg/ml GEM, and 2 μM MTX. (C) The concentrations of GEM and ADR used for MDA-MB-231 and MCF-7 cells were 20 μg/ml and 20 μM, respectively. (D) Downregulation of Akt and upregulation of p38 phosphorylation correlated with PARP cleavage induced by serum starvation, TNF-α, and UV irradiation. 231-E1A cells were serum starved [serum(−)], exposed to TNF-α (5 ng/ml), or UV irradiated (6 J/cm^2) while parental MDA-MB-231 cells were exposed to 10-times-higher doses of TNF-α (50 ng/ml) and UV radiation (60 J/cm^2). Both the attached cells and cells in suspension were collected, if not specified. W, whole-cell lysate with both attached and suspended cells; A, attached cells only; D, detached or floated apoptotic cells.

regulation of Akt activation and upregulation of p38 activation may also represent a general cellular mechanism in response to different apoptotic stimuli.

The physiological regulation of p38 activity by Akt is through ASK1 and MEKK3, the upstream kinases of p38. The above results suggest that both downregulation of Akt and upregulation p38 activities are involved in E1A-mediated sensitization to apoptosis. We noticed that reduced Akt phosphorylation occurs before enhanced p38 phosphorylation in the kinetic study of E1A-mediated sensitization to paclitaxel-induced PARP cleavage (Fig. 2A). We therefore asked whether Akt may act upstream of p38. To this end, Akt activity was blocked by either a specific PI3K inhibitor, wortmannin, or a genetic method to knock out Akt expression. Blocking Akt activation with wortmannin in MDA-MB-231 cells resulted in decreased Akt phosphorylation and increased p38 phosphorylation (Fig. 5A). And when Akt phosphorylation was recovered, the p38 phosphorylation was reduced again (8- to 24-h time points). These results indicate that Akt phosphorylation was required for repressing p38 activation, suggesting that the former is upstream from the latter. This conclusion was further supported by the study with Akt1-knockout MEFs and myr-Akt1-transfected stable cells. We observed that the level of phosphorylated p38 was increased in Akt1$^{-/-}$ MEFs compared with that in Akt$^{+/+}$ and Akt$^{+/-}$ MEFs. Furthermore, the phospho-p38 protein was undetectable in the Akt constitutively activated myr-Akt1 stable cells (Fig. 5B). These results indicate that Akt is able to inhibit p38 activity.

In an attempt to determine how Akt regulates p38, we sought to determine whether Akt is physically associated with p38 by using coimmunoprecipitation experiments. We did not detect p38 in immunoprecipitated Akt samples (Fig. 5C) or Akt in immunoprecipitated p38 samples (data not shown), suggesting that Akt and p38 were not directly associated under

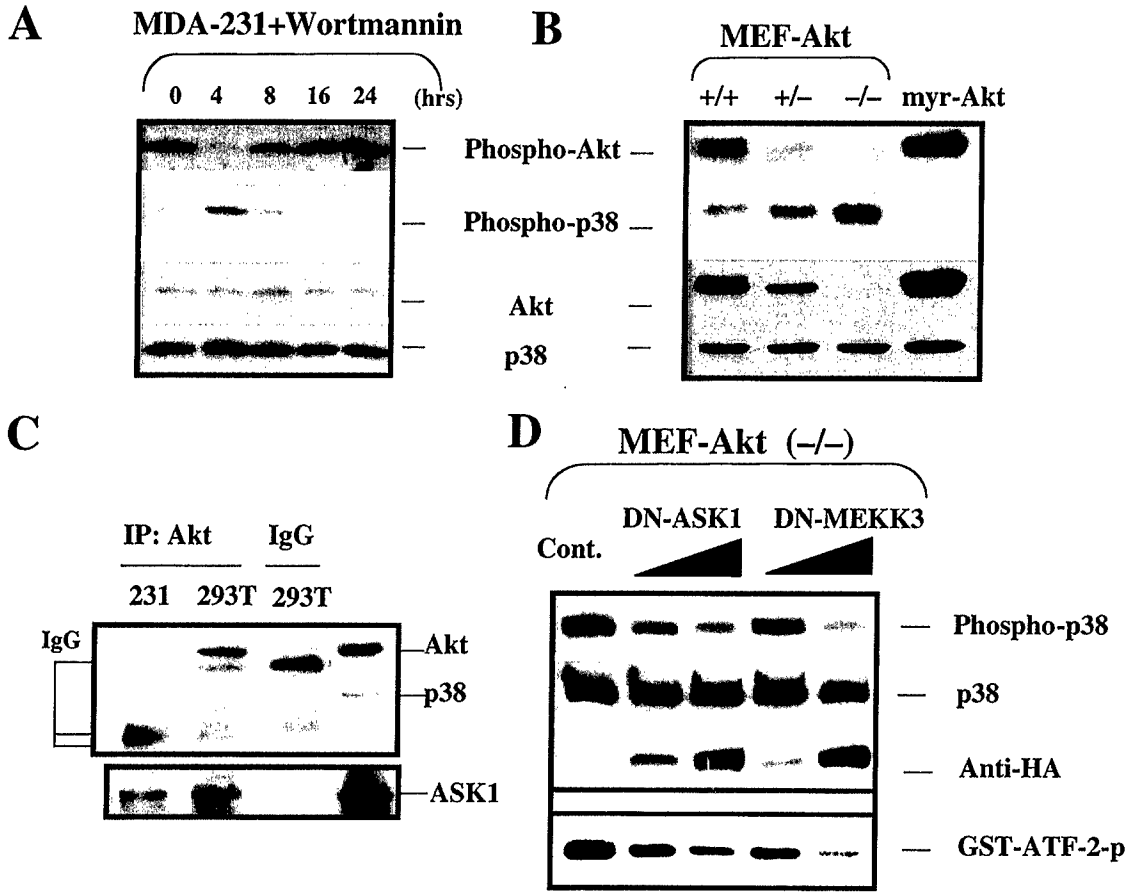


FIG. 5. Physiological regulation of Akt and p38 pathways. (A) Stable E1A-expressing or parental MDA-MB-231 cells were serum starved for 24 h before exposure to 20.0 μM 0.1 μM wortmannin. (B) Expression of phospho-p38 and phospho-Akt in Akt1 knockout MEFs and myr-Akt-transfected Rat1cells. (C) CA-Akt was transiently transfected into both MDA-MB-231 and 293T cells. The cells were lysed after transfection, and Akt was immunoprecipitated (IP). Western blot analyses of Akt, ASK1, and p38 interaction in 293T cells and MDA-MB-231 cells were performed. (D) $Akt^{-/-}$ MEFs were grown in six-well plates for 24 h and then transiently transfected by FuGENE 6 liposome (catalog no. 1 814 443; Roche Molecular Biochemicals, Indianapolis, Ind.) at a 3:1 ratio with either HA-tagged DN-ASK1 or HA-tagged DN-MEKK3 cDNA in the amounts of 1 and 10 μg, respectively. Cells were grown for another 36 h and were then harvested and analyzed for p38 kinase activity with ATF-2 as the substrate. The expressions of phospho-p38, total p38, and the HA tag were also detected by the respective antibodies. IgG, immunoglobulin G; Cont., control cells transfected with pcDNA3 plasmid DNA.

the conditions we used. A recent report demonstrated that ASK1 is a substrate of Akt (28), and ASK1 has been shown to be an upstream kinase of p38 (23, 51), suggesting that Akt may indirectly regulate p38 activity through ASK1. Indeed, we also detected that ASK1 was coimmunoprecipitated with Akt in our experimental system (Fig. 5C). To test whether Akt can down-regulate p38 activation through the repression of p38 upstream kinases, such as ASK1, we blocked the activity of either ASK1 or MEKK3, both of which are p38 upstream kinases that can be inactivated by Akt (17, 28), by using a kinase-dead, DN mutant of ASK1 (DN-ASK1) or MEKK3 (DN-MEKK3). As expected, blockade of either ASK1 or MEKK3 activity by DN-ASK1 or DN-MEKK3 repressed p38 phosphorylation and its kinase activity, as measured by phosphorylation of ATF2 in a dose-dependent manner in $Akt1^{-/-}$ MEFs (Fig. 5D), suggesting that Akt inhibits p38 activation through repression of ASK1 and/or MEKK3 activation.

p38 inactivation is associated with Akt activation in human cancer. The above results suggest that Akt acts upstream of p38 and blocks p38 activation. Because activation of Akt is a common phenomenon in different types of human cancers, we asked whether p38 inactivation is also a common phenomenon in human cancer cells and correlates with Akt activation. To test whether p38 inactivation was accompanied by Akt activation in human tumor tissues in vivo, we utilized tissue array slides to screen phospho-p38 and phospho-Akt expression in tumor tissues of different origins and normal or parallel normal organ tissues. We found that the phospho-Akt level was dramatically higher while phospho-p38 was undetectable in most of the cancer tissues obtained from different types of solid tumors, such as breast, lung, liver, bile duct, gastric, colorectal, renal cell, ovarian, and uterine cancers; malignant lymphoma; and Schwannoma. In contrast, the intensity of phospho-p38 protein staining was relatively strong while that of phospho-Akt staining was very weak in normal organs and parallel healthy tissues. Representative data on the expression of phospho-p38 and phospho-Akt in healthy versus tumor tissues obtained from the breast, lung, liver, and biliary duct are shown

in Fig. 6A. By screening a panel of breast, ovarian, prostate, pancreatic, and colorectal cancer cell lines with phosphospecific antibody against p38 or Akt, we also observed a correlation between enhanced Akt phosphorylation and reduced p38 phosphorylation in these human cancer cell lines (data not shown). These data support the hypothesis that p38 activity is repressed in different types of human cancer, which is associated with enhanced Akt activation.

To test whether repression of p38 and activation of Akt also correlate with tumor stage in human cancer, we analyzed 10 healthy breast tissue samples (including 2 healthy nipple and 8 healthy breast tissues) and 50 cases of breast cancer at different stages on Histo-Array slides. These include 4 cases at stage I (T1), 21 cases at stage II (T2), 20 cases at stage III (T3), and 5 cases at stage IV (T4). Among the 25 cases of early stage tumors (T1 and T2), 12% of them are negative and half of them are weakly positive for phospho-Akt staining. In the advanced late-stage tumor samples, all of them are positive and more than 80% of them are moderately to strongly positive for phospho-Akt staining (Fig. 6B), whereas the relative intensity of phospho-p38 staining was inversely correlated with the tumor stage (Fig. 6B). In the early stage tumor samples, only one-third of them (36%) are positive for phospho-p38 staining and only 1 of 25 cases is moderately positive for phospho-p38. In the late-stage tumor samples (T3 and T4), 80% of them are negative for phospho-p38 staining and the rest are weakly positive for phospho-p38 staining (Fig. 6B). Comparing the phospho-p38 staining and phospho-Akt staining in the advanced late-stage tumor samples, we observed an inverse correlation between the intensity of strong phospho-Akt staining versus weak phospho-p38 staining ($P < 0.0001$) (Fig. 6C). Phosphorylated p38 could be detected in most of the healthy organ tissues but not in most of the cancer tissues or cell lines, indicating that p38 inactivation is also a common event in human cancer cells with Akt activation.

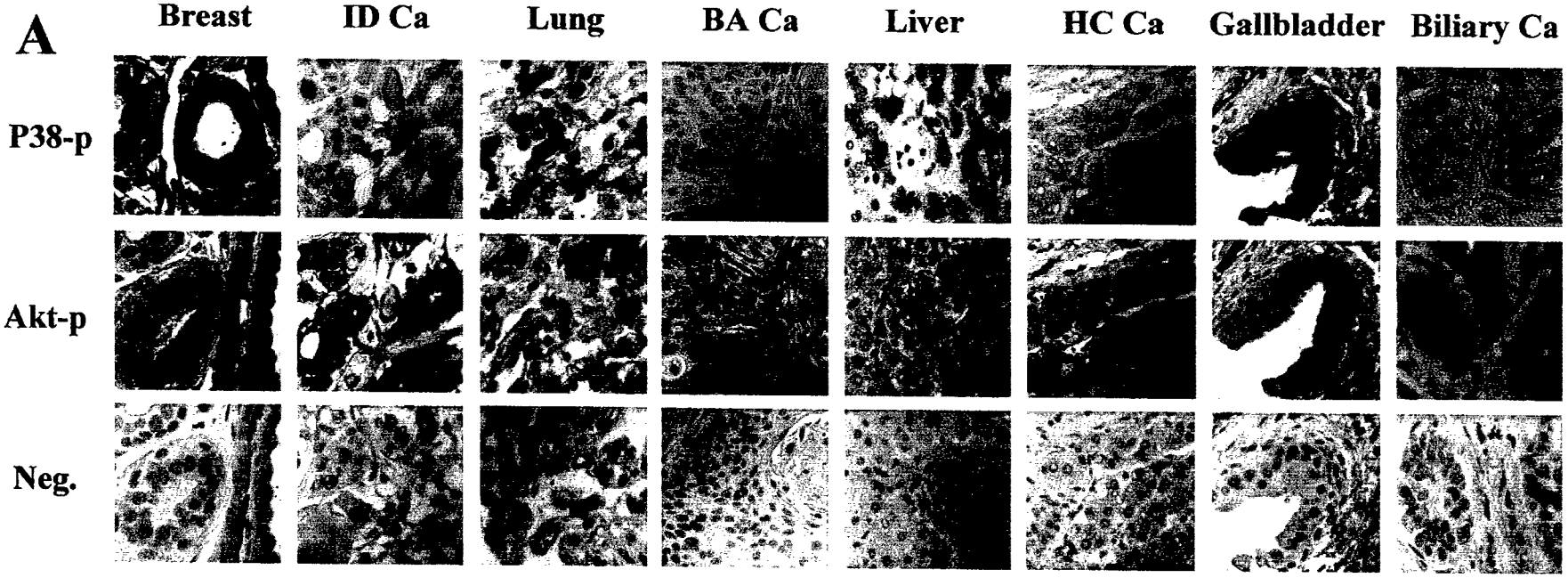
The CR2 domain of E1A is required for downregulation of Akt phosphorylation and chemosensitization. The above results suggested that Akt regulated p38 activation in both physiological and pathological conditions, which indicates that E1A-mediated downregulation of Akt activity and upregulation of p38 activity are accompanied events, i.e., by repression of Akt activation, E1A enhanced p38 activity and sensitized cells to drug-induced apoptosis. To lay further genetic support for these conclusions, we proposed to map the domain(s) of E1A that is responsible for downregulation of Akt activity and demonstrate that the same domain is also critical for E1A-mediated upregulation of p38 and sensitization to drug-induced apoptosis. It is known that among the three conserved domains (CR) of E1A, CR1 and CR2 are associated with E1A-mediated sensitization to apoptosis (46). Therefore, we established E1A functional domain deletion mutation stable cells in MDA-MB-231 cells, including wild-type E1A, and deletion mutations of CR1 (ΔCR1) and CR2 (ΔCR2) (Fig. 7A). We found that deletion mutations of the CR2 domain dramatically disrupted E1A's ability to downregulate Akt kinase activity, eliminated E1A-mediated upregulation of p38 kinase activity, and remarkably repressed E1A-mediated sensitization to paclitaxel-induced apoptosis (Fig. 7B and C) while the CR1 domain mutant only slightly affected E1A-mediated chemosensitization and downregulation of Akt and upregulation of p38 activities (Fig. 7B and C). These results indicate that the same CR2 domain required for downregulation of Akt is also required for upregulation of p38 and sensitization to drug-induced apoptosis and thus supports the conclusions that Akt represses p38 activity and that E1A, by downregulation of Akt activity, enhances p38 activation and sensitizes cells to anticancer drug-induced apoptosis.

DISCUSSION

The present study shows that the activity of p38 is regulated by Akt and is deregulated partly due to Akt activation in human cancer. Activation of Akt antagonizes p38 activation while inactivation of Akt results in p38 activation. The adenoviral protein E1A, by downregulation of Akt activity, enhanced p38 activation and sensitized cells to apoptosis induced by different apoptotic stimuli. It is known that p38 participates in the regulation of apoptotic cell death through transcriptional upregulation of proapoptotic gene expression, such as Fas ligand (11, 13, 20, 38). p38 is also involved in negative regulation of cell growth, as it represses cyclin D1 expression and regulates the G_2-M transition through the regulation of cdc25 protein phosphatase and p53 protein (6, 42). Recently, inactivation of p38 has been shown to contribute to the development of human cancers by suppressing p53 activation (7), suggesting a tumor-suppressive function of p38. In contrast, Akt is known to upregulate the cyclin D1 expression while repressing Fas ligand expression and p53 stabilization (39, 50). Akt is also involved in regulation of the G_2-M transition (26, 40, 47). Thus, Akt may functionally antagonize the p38 effect on cellular processes ranging from cell cycle progression to cell death, though some cell types may respond differently (3, 8, 17, 19, 44). However, the regulation between the Akt and p38 pathways is still unclear in the literature, and the present study provides a link between activation of Akt and inactivation of p38. As discussed above, Akt positively regulates cell growth but negatively regulates cell death while p38 positively regulates cell death but negatively regulates cell growth. Given the results we obtained in $Akt^{-/-}$ MEFs (Fig. 5B and D) and the fact that Akt directly phosphorylates and negatively regulates the activation of ASK1 and MEKK3, the upstream kinase of p38 (17, 28), we propose that Akt may repress p38 activation through the phosphorylation and inactivation of ASK1 or MEKK3 and that inactivation of Akt may result in p38 activation through the release of ASK1 and/or MEKK3 activity. Because either DN-ASK1 or DN-MEKK3 sufficiently repressed enhanced p38 phosphorylation in $Akt1^{-/-}$ MEFs (Fig. 5D), it also suggests that both ASK1 and MEKK3 are involved in Akt-mediated inactivation of p38. Regulation of p38 activity by Akt2 through ASK1 was also demonstrated recently by Yuan et al. in their report on cisplatin-induced apoptosis (57), suggesting that both Akt1 and Akt2 may use a similar mechanism to inactivate p38.

We have shown that activation of p38 follows inactivation of Akt (Fig. 2 and 4B to D) when cells underwent apoptosis, suggesting that the pro- and antiapoptotic signals may integrate each other to prepare cells to commit suicide. The relative Akt and p38 activity may determine a cell's response to apoptotic stimuli, as they can be observed in E1A-mediated sensitization to apoptosis induced by serum starvation, TNF-α,

A



6844

B

Immunohistochemistry analysis of phospho-Akt and phospho-p38 expression in human breast cancer tissues

	Phospho-Akt		Phospho-p38	
T Stage	T1~T2 (n=25)	T3~T4 (n=25)	T1~T2 (n=25)	T3~T4 (n=25)
−	3 /25 (12%)	0 /25 (0)	18/25 (64%)	20/25 (80%)
+	11/25(44%)	4 /25 (16%)	8 /25 (32%)	5 /25 (20%)
++	6 /25 (24%)	11/25(44%)	1 /25 (4%)	
+++	6 /25 (24%)	10/25(40%)		

C

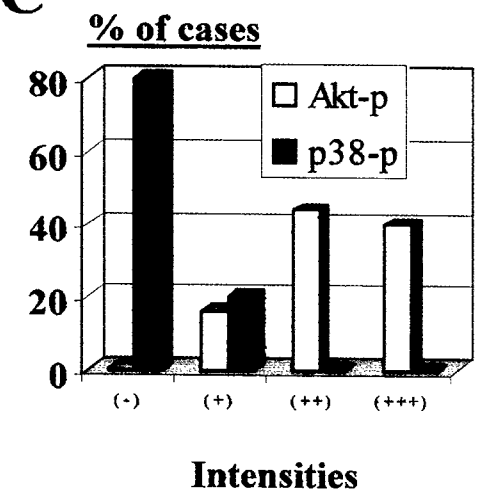


FIG. 6. Inactivation of p38 associated with Akt activation in human cancer. (A) Immunohistochemical staining of phospho-p38 and phospho-Akt in different types of human cancer tissues in the Histo-Array slides. Ca, carcinoma; ID Ca, infiltrating ductal carcinoma; BA Ca, bronchoalveolar carcinoma; HC Ca, hepatocellular carcinoma. (B) Intensity of phospho-Akt and phospho-p38 stained in the early and late stages of breast cancer. The staining intensity of phospho-Akt is significantly greater in late stage (stage III and IV) breast cancer while positive phospho-p38 staining is predominant in healthy breast epithelial cells and early stage (stage I and II) breast cancer ($P < 0.001$ for both). (C) Inverse correlation between phospho-Akt and phospho-p38 staining in late stage breast cancer tissue samples.

UV irradiation, and different categories of chemotherapeutic drugs (Fig. 4B to D). Expression of E1A may shift the balance of the pro- and antiapoptotic signals by repressing Akt activity and enhancing p38 activity, thereby favoring the proapoptotic signal. Additional approaches could also be used by E1A to shift the intracellular signal integration to favor a proapoptotic signal, such as activation of p53 and caspase proenzymes, but these pathways may not contribute to the present study. For example, p53 and p14ARF were deleted in MDA-MB-231 cells and we did not detect any change in the expression level of p53 or p14ARF in E1A versus parental control cells (data not shown), i.e., the p53-dependent mechanisms may not contribute to E1A-mediated sensitization to apoptosis in the present study. Although expression of E1A by infection of cells with either retroviral or adenoviral vector resulted in the accumulation of caspase proenzymes, such as caspase 3, 7, 8, and 9, by a direct transcriptional mechanism through enforced E2F-1 release in normal diploid human fibroblasts (IMR90) (37). We did not observe a consistent increase of these caspase proenzymes in the E1A stable cells established in human cancer cells with epithelial origin (Fig. 1D). In addition, sensitization to the DNA damage agent adriamycin-induced apoptosis by E1A is dependent on p53 status in normal fibroblasts (37) while E1A dramatically sensitized the adriamycin therapeutic effect in ovarian cancer SKOV3.ip1 (5) and breast cancer MDA-MB-231 cells (Fig. 4A), which do not express functional p53. The discrepancy between normal diploid fibroblasts and epithelial carcinoma cells in E1A-mediated sensitization to apoptosis may reflect the nature of the intrinsic difference between normal fibroblasts and carcinoma cells. However, downregulation of Akt activity by E1A was also observed in E1A-mediated sensitization to cisplatin in human normal IMR90 fibroblasts (54). Thus, targeting the key oncogenic survival factor Akt may represent a critical mechanism for E1A-mediated sensitization to anticancer drug-induced apoptosis in human cancer cells and normal fibroblasts as well.

E1A has been shown to facilitate cytochrome *c* release from the mitochondria, which also contributes to E1A-mediated sensitization to anticancer drugs. However, the mechanism by which E1A facilitates cytochrome *c* release is unclear (14). Although we did not test whether E1A expression affected Bax translocation, which may also facilitate cytochrome *c* release, E1A expression or treatment with paclitaxel did not affect the levels of Bax protein in our system (Fig. 2A). Akt is known to play an important role in maintaining mitochondrial integrity and inhibiting the release of cytochrome *c* (11, 27). Overexpression of Akt confers resistance to paclitaxel by inhibiting paclitaxel-induced cytochrome *c* release (41). However, p38 is also involved in regulation of cytochrome *c* release (2). Therefore, it is possible that E1A may alter mitochondrial potential by downregulating Akt and upregulating p38, thereby facilitating the release of cytochrome *c* upon treatment with chemotherapeutic drugs, such as paclitaxel. Thus, downregulation of key survival factor Akt activity and subsequent upregulation of a proapoptotic factor p38 activity by E1A may constitute a fundamental approach for E1A-mediated sensitization to apoptosis.

The mechanisms underlying E1A-mediated downregulation of Akt activity are not yet clear. Obviously, E1A-mediated downregulation of Her-2/neu and/or Axl may contribute to

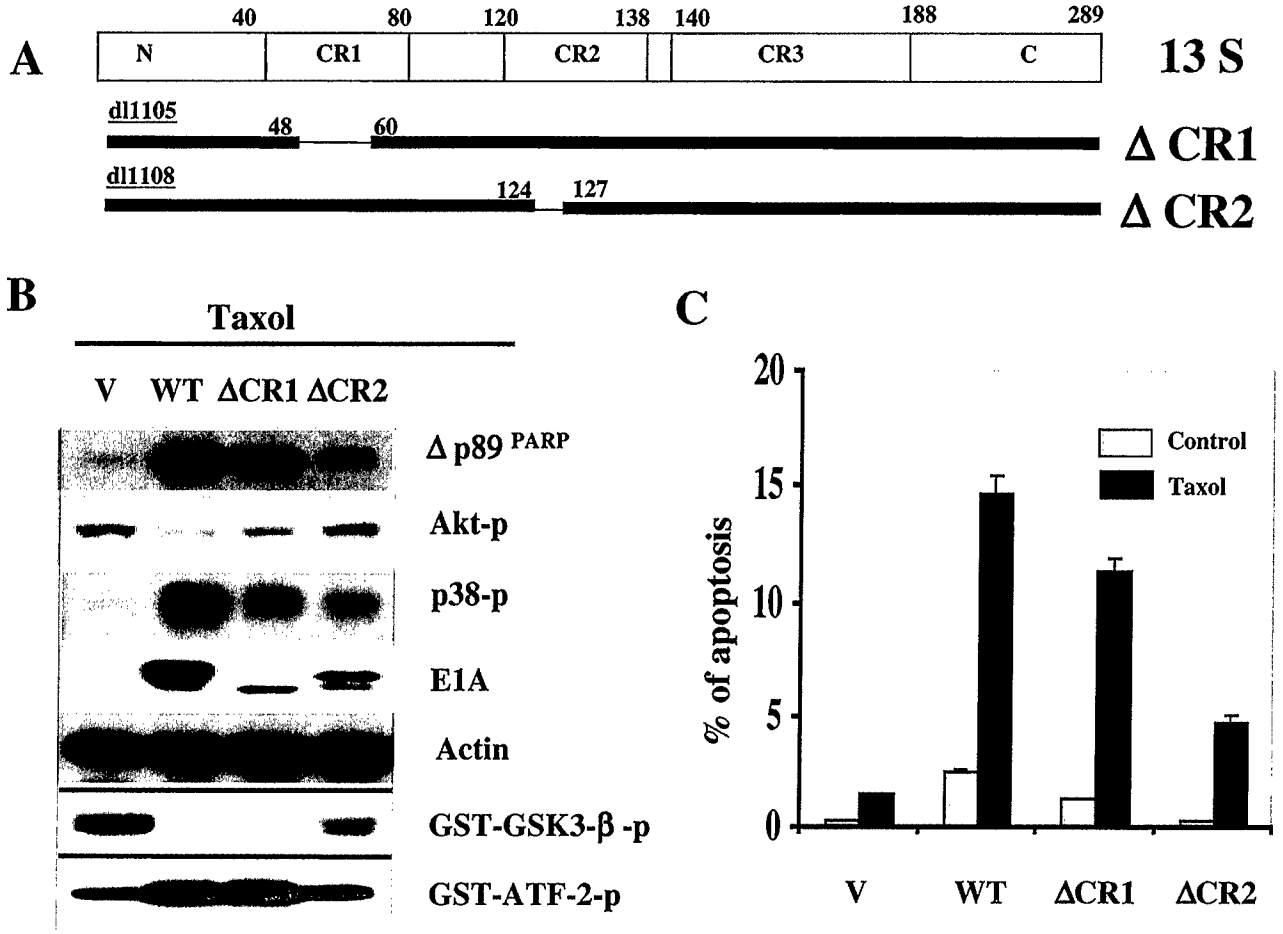


FIG. 7. The CR2 domain of E1A is required for downregulation of Akt and sensitization to drug-induced apoptosis. (A) Domain structure and map for deletion mutation of CR1 and CR2. (B) Akt and p38 kinase activity were measured, and Western blot analysis of PARP cleavage, phospho-Akt, phospho-p38, or E1A expression was performed in different domain deletion mutant stable cells. Actin was used as a loading control. (C) FACS analysis of apoptosis of wild-type (WT) E1A and different domain deletion mutant E1A stable cells with or without treatment with 0.01 μM paclitaxel for 21 h. V, pSV-neo vector-transfected stable cells.

reduced Akt activation, as activation of either Her-2/neu or Axl leads to PI3K-Akt kinase activation and downregulation of Her-2/neu and/or Axl also contributes to E1A-mediated sensitization to apoptosis (21, 29, 30, 58). However, downregulation of Akt activity by E1A may not necessarily depend on E1A-mediated downregulation of Her-2/neu and/or Axl, because both MDA-MB-231 and MCF-7 cells are low Her-2/neu-expressing cells and MCF-7 cells have an undetectable expression level of Axl (35). In addition, deletion mutation of the CR2 domain affects E1A-mediated downregulation of Akt (Fig. 7B), but it has no effect on E1A-mediated transcriptional repression of Her-2/neu (9). Thus, in addition to the Her-2/neu-dependent pathway, a Her-2/neu-independent pathway must exist for E1A to mediate downregulation of Akt activity leading to sensitization to apoptosis. In addition, overexpression of Her-2/neu or activation of Akt also leads to p53 destabilization (50, 59), suggesting that downregulation of Akt activity by E1A may constitute an alternative pathway for stabilization of p53. Like Her-2/neu and p53, Akt also plays a critical role in the regulation of apoptotic cell death and the development of human cancer. Therefore, E1A-mediated downregulation of Akt and upregulation of p38 activities may have general implications for E1A-mediated tumor suppression and sensitization to apoptosis.

ACKNOWLEDGMENTS

We thank Richard R. Vaillancourt (The University of Arizona College of Pharmacy, Tucson, Ariz.) for providing the HA-tagged kinase-dead DN mutant MEKK3 (MEKK3-KM) and Philipp E. Schere (Albert Einstein College of Medicine of Yeshiva University) for providing the IPTG-inducible DN-p38 constructs. We also thank N. Hay (University of Illinois at Chicago) for providing us with a panel of Akt-knockout MEFs and myr-Akt stable cells. We acknowledge Zheng Huang's technical support for staining and reading of Histo-Array slides. We thank Stephanie Miller for reading and editing the manuscript.

This work was supported by grant RO1-CA58880 and the SPORE grant for ovarian cancer research from the National Institutes of Health (to M.-C.H.) and by grant DAMD17-01-1-0300 from the U.S. Department of Defense Army Breast Cancer Research Program (to Y.L.).

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Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by a non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer*.

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Running title: Enhanced paclitaxel efficacy by systemic delivery of the E1A gene

Keywords: breast cancer, xenograft; E1A, gene therapy; systemic gene delivery; non-viral delivery

* This work was supported in part by NIH Grant RO1-CA58880 and the SPORE grant for ovarian cancer research P50 CA83639 from the National Institutes of Health (to M.-C. H.) and DAMD17-01-1-0300 from the United States Department of Defense Army Breast Cancer Research Program (to Y. L.).

ABSTRACT

Paclitaxel (Taxol) is a promising frontline chemotherapeutic agent for the treatment of human breast and ovarian cancers. The adenoviral type 5 E1A gene has been tested in multiple clinical trials for its anti-cancer activity. E1A has also been shown to sensitize paclitaxel-induced killing in E1A-expressing cells. Here we show that E1A can sensitize paclitaxel–induced apoptosis in breast cancer cells in a gene therapy setting by an orthotopic mammary tumor model. We first showed that expression of E1A enhanced *in vitro* paclitaxel cytotoxicity, as compared to the control cells. We then compared the therapeutic efficacy of paclitaxel between orthotopic tumor models established with vector-transfected MDA-MB-231 (231-Vect) versus 231-E1A stable cells, using tumor weight and apoptotic index (TUNEL assay) as the parameters. We found paclitaxel was more effective in shrinking tumors and inducing apoptosis in tumor models established with stable 231-E1A cells than the control 231-Vect cells. We also tested whether E1A could directly enhance paclitaxel-induced killing in nude mice, by using a nonviral, surface-protected cationic liposome to delivery E1A gene via the mouse tail vein. We compared the therapeutic effects of E1A gene therapy with or without Taxol chemotherapy in the established orthotopic tumor model of animals inoculated with MDA-MB-231 cells, and found that a combination of systemic E1A gene therapy and paclitaxel chemotherapy significantly enhanced the therapeutic efficacy and dramatically repressed tumor growth ($P < 0.01$). In addition, survival rates were significantly higher in animals treated with combination therapy than in the therapeutic control groups (both $P < 0.0001$). Thus, the E1A gene therapy indeed

enhance the sensitivity of tumor cells to chemotherapy in a gene therapy setting and, the current study provides preclinical data to support combination therapy between E1A gene and chemotherapy for the future clinical trials.

INTRODUCTION

Amplification and overexpression of *Her-2/neu* (or c-erbB-2) oncogene have been found in approximately 25% of human breast carcinomas and associated with poor prognosis (1). Using *Her-2/neu* as a target for the development of therapeutic agents proves to be an effective approach. For example Herceptin, a humanised monoclonal antibody against *Her-2/neu*, have demonstrated its efficacy by either Herceptin alone or in combination with cisplastin as a first-line therapy for the treatment of breast cancer patients with advanced disease whose tumors overexpressed *Her-2/neu* (2, 3). We have previously reported that the adenoviral type-5 E1A gene product also repressed *Her-2/neu* oncogene overexpression and suppressed the tumorigenic and metastatic potential of *Her-2/neu* transformed cells (4).

The adenoviral type-5 and type-2 E1A were reported originally as an oncogene, which could cooperate with other viral and cellular oncogenes to transform primary culture cells but not established cell lines, distinct from the type-12 E1A, a potent oncogene that can transform established cell lines (5). However, E1A has not been associated with human malignancies despite extensive studies trying to identify such a link. Instead, E1A was shown to suppress experimental metastasis of rodent cells transformed by the *ras* oncogene (6-8) and by the *Her-2/neu* oncogene (9) and metastasis of certain human cancer cell lines (10). In addition, increasing experimental data indicate that E1A was able to inhibit the tumorigenicity of the tranformed rodent cells as well as of the human cancer cell lines (5, 10). Therefore, based on its ability to suppress both tumorigenicity and metastasis, E1A has been considered as a tumor suppressor gene (5, 11-13) and translated into multiple clinical trials (14-16).

In addition to the tumor suppressor activities, expression of the E1A gene in stably transfected normal fibroblast and human cancer cells has also been shown to increase sensitivity to the *in vitro* cytotoxicity of several anticancer drugs, such as etoposide and cisplatin in normal fibroblasts and sarcoma cells, Adriamycin in colon and hepatocellular carcinoma cells, Gemcitabine in hepatocellular and breast cancer cells, and paclitaxel in breast and ovarian cancers (17-27). Our previous studies demonstrated that E1A can sensitize paclitaxel-induced cell killing in *Her-2/neu*-overexpressing cancer cells (22, 23). We did not observe E1A-induced paclitaxel sensitization for *Her-2/neu* low-expressing cells under the same condition, in which the paclitaxel concentration is too high to observe sensitization effect in the *Her-2/neu* low-expressing cells (please see discussion later) (22). However, in our recent study, we found that expression of E1A in *Her-2/neu* low-expressing breast cancer MDA-MB-231 and MCF-7 cells sensitized cells to different categories of anti-cancer drug-induced apoptosis at a varying extent, these drugs including paclitaxel, cisplatin, Adriamycin, Gemcitabine, and methotrexate (26). Since these drugs exert its anti-cancer activity through different mode of actions, we showed that E1A-mediated sensitization to different types of anti-cancer drug-induced apoptosis was achieved through a general cellular pathway that regulate the cell survival and cell death signalings (26). In the current study, we further examine the E1A-induced chemosensitization for the *Her-2/neu* low-expressing cancer cells in a mammary tumor model using the newly developed systemic nonviral delivery system SN, which contains a surface-protection polymer to stabilize the liposome-DNA particles for i.v. injection (28-30). We tested if combination of E1A gene therapy with paclitaxel chemotherapy will enhance the cytotoxicity and anti-tumor effect of paclitaxel in an orthotopic breast

cancer xenograft established by inoculation of low *Her-2/neu*-expressing MDA-MB-231 cells in nude mice. We found that combination of systemic E1A gene therapy with paclitaxel significantly enhanced paclitaxel-induced apoptosis and prolonged animal survival rates in the orthotopic model *in vivo*. Thus, the current study provides further experimental support for combination therapy between E1A gene therapy and paclitaxel chemotherapy.

Materials & Methods

Cell lines

Human breast cancer MDA-MB-231 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies Inc., Rockville, MD) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines were established as previously described (31).

Paclitaxel

A stock solution of paclitaxel (Taxol, Bristol-Myers Squibb Co., Princeton, NJ) was stored at -80°C before use. For *in vitro* use, paclitaxel was diluted in serum-free medium at the required concentration. For *in vivo* use, paclitaxel was diluted in normal saline (NS) to a dose of 10 mg/kg in 150 μl per injection, once a week for 6 weeks.

Formulation

The gene delivery system, termed SN, was described by Zou et al (28, 29). The DNA was entrapped in the SN-liposome after the thin-lipid film was hydrated and extruded through a filter with 0.2-μm-diameter pores (Gelman Sciences; Ann Arbor, MI).

MTT assay

The metabolic conversion of tetrazolium salt (MTT) to formazan was used to indirectly measure the number of viable cells after exposure to Taxol. Cells (3 x 10^3/well) were seeded in triplicate in 96-well culture plates in 0.2 ml of culture medium and allowed to adhere for 24 h. After exposure to Taxol for indicated time periods, 20 μl of MTT was added to each well. Cells were cultured for an additional 2 h, and 100 μl of extraction buffer (20% SDS in 50% *N,N*-dimethyl formamide, pH 4.7) was added to the culture medium. The cells were incubated overnight at 37 °C, and the plate absorbency was measured at 570 nm.

Propidium iodide staining and FACS analysis

Samples of 2 x 10^6 cells were collected, washed once with phosphate-buffered saline (PBS), and fixed with 70% ice-cold ethanol overnight. After fixation, cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing 50 μg/ml of propidium iodide (Sigma, St. Louis, WA). The staining was performed at 4 °C for at least 30 min, and samples were analyzed using a FACScan (Becton-Dickinson, San Jose, CA) in the core facility at The University of Texas M. D. Anderson Cancer Center.

Athymic Nude Mice

Four to 6-week-old female athymic BALB/c-*nu/nu* mice were purchased from Charles River Laboratories (Wilmington, MA). The animals were allowed to acclimate for 7 days before the study initiation. All of the animals were housed under pathogen-free conditions, and were given water and chow *ad libitum*. Animal care and use were in accordance with Institutional and NIH guidelines.

Establishment of orthotopic breast cancer model and systematic E1A gene therapy

in nude mice

MDA-MB-231 cells (1 x 10 ^{6} cells/0.1 ml) or 231-E1A cells (2 x 10^6 cells/0.1 ml) were subcutaneously injected into the mammary fat pad (m.f.p.) of female, athymic mice. Tumors were allowed to develop for 21 days and mice were then randomly grouped and treated with SN-liposome alone (SN, *i.v.*), Taxol alone (*i.p.*, 10 mg/kg/injection, once a week for 6 weeks), E1A alone (*i.v.*, 15 μg/mouse/injection, injection into mouse tail vein twice a week for 6 weeks), or E1A (*i.v.*, 15μg/mouse/injection) plus Taxol (*i.p.*, 10 mg/kg/injection, 24 hours after E1A injection, for 6 weeks). Both maximum and minimum diameters of the resulting tumors were measured twice a week using a slide caliper. Tumor volumes were calculated by assuming a spherical shape and using the formula, volume = $4/3r^3$, where r = 1/2 of the mean tumor diameter measured in two dimensions. Mice were sacrificed when their tumors were larger than 2 cm in diameter otherwise mice will keep growing and subject for survival rate analysis.

Immunohistochemical Analysis

To evaluate expression of E1A in stable MDA-MB-231 cells, cells in exponential growing were harvested and washed with PBS twice and then cytospuned. Cytospun cells were fixed for 10 min in 4% formaldehyde (Sigma), washed with PBS, dried, and stored at -80°C or subject to immunohistochemical analysis. To evaluate expression of E1A protein level in tumor tissues in vivo, stable E1A expressing cells or control vector cells were inoculated into m.p.f. of nude mice. After a palpable tumor was formed, approximately 4 to 6 weeks, tumor dissected and fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded sections were then pretreated with dewax and rehydrated. They were washed in xylene and rehydrated through a graded series of

ethanol and redistilled water. The paraffin sections or cytospun slides were incubated with E1A M73 monoclonal antibody (Oncogene Science, Inc., Cambridge, MA) diluted 1:20. The slides were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) or biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:200 in PBS. The slides were then incubated with an avidin-biotin-peroxidase complex (Vector Laboratories), and the peroxidase-catalyzed product was visualized with 0.125% aminoethyl carbazole chromogen buffer (Sigma Chemical Co.).

***In vivo* apoptotic (TUNEL) assay**

For *in vivo* apoptotic assay, tumors were fixed in 10% formalin and embedded in paraffin blocks as described above. Tissue sections were incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl, pH 7.4 to 8.0, for 15 min at 37°C), permeabilized in 0.1% Triton-X-100 in 0.1% sodium citrate, and then labeled with the TUNEL reaction mixture (Boehringer Mannheim, Indianopolis, Indiana) according to the manufacturer's protocol. Briefly, biotinylated nucleotide mix and TdT enzyme were added and incubated for 1 h at 37°C; slides were washed in PBS, blocked in hydrogen peroxide, and incubated in streptavidin horseradish peroxidase. The slides were developed in 3,3'-diaminobenzidine and counterstained with hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. The apoptosis index was defined by the percentage of brown cells among the total cells of each sample. Ten fields with >200 cells in each were randomly counted for each sample.

Results

E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis *in vitro*

To test whether E1A can sensitize cells to paclitaxel-induced apoptosis in low *Her-2/neu*-expressing cells in cell culture *in vitro*, we treated MDA-MB-231 (p53 mutant) and MCF-7 (p53 wild type) human breast cancer cell lines and their E1A-expressing stable cells with different dosages of paclitaxel and performed MTT cytotoxicity assays. We found that stable expression of wild-type E1A enhanced sensitivity to paclitaxel-induced killing in MDA-MB-231 cells in a dose dependent manner. Compared with their IC_{50} dosage, the E1A-expressing stable cells were ten times more sensitive than that of the paclitaxel-treated parental or vector-transfected control cells (Figure 1A). Stable expression of E1A also enhanced the sensitivity of MCF-7 cells to paclitaxel, although to a lesser extent. Interestingly, a revertant of the MCF-7 clone that lost its E1A expression during long-term *in vitro* cell culture also lost its sensitivity to paclitaxel (Figure 1B). Three additional independent E1A stable clones from each cell line were analyzed, all of which expressed high levels of E1A protein and showed similar sensitivity to paclitaxel (data not shown).

To address whether apoptosis was involved in E1A-mediated sensitization to paclitaxel, we examined the presence of poly (ADP-ribose) polymerase (PARP) cleavage as an apoptotic cell death marker. We detected a dose-dependent increase of PARP cleavage in E1A-expressing MDA-MB-231 (231-E1A) and MCF-7 (MCF-7-E1A) cells upon treatment with 0.1 μM to 0.001 μM paclitaxel. In parental and 231-Vect cells, however, PARP cleavage was observed only upon treatment with 0.1 μM paclitaxel, while in the revertant MCF-7 cell clone, it was not detected (Insert, Figure 1A and B).

Fluorescence-activated cell sorting (FACS) analysis also supported that E1A enhanced paclitaxel-induced apoptosis, as demonstrated by an increased proportion of sub-G1-phase cells in E1A-expressing cell lines (Figure 1C). Thus, E1A-mediated sensitization to paclitaxel in breast cancer MDA-MB-231 and MCF-7 cells consistent to the previous report for E1A-mediated sensitization to multiple chemotherapeutic drugs (26, 32).

Expression of E1A enhances paclitaxel-induced anti-tumor effect *in vivo*

To test whether E1A also mediated sensitization to paclitaxel *in vivo*, we inoculated stable E1A-expressing (231-E1A) and vector-transfected (231-Vect) MDA-MB-231 cells into mouse m.f.p. In our pilot study, we inoculated same amount of MDA-MB-231 and 231-E1A cells into mouse m.f.p and observed that few of the animals with inoculation of the 231-E1A cells developed tumor and even though in some cases, the tumors are developed, but they are much smaller than those of the MDA-MB-231 cells (data not show). Therefore, in order to ensure tumor formation with comparable size in 231-E1A-inoculated animals, twice amount of 231-E1A cells were inoculated. When tumor established, animals were then treated with or without paclitaxel, via intra-peritoneal injection, once a week, for four consecutive weeks. Mice were grown for another six weeks and were then sacrificed. Tumor tissues were collected and measured. The mean tumor weight of animals inoculated with 231-E1A cells is significantly lower than that of animals inoculated with 231-Vect cells (Figure 2A, $P < 0.01$), even though twice amount of cells were original inoculated in 231-E1A group. The result supported the previous reports that expression of E1A alone had a significant effect on repression tumor growth *in vivo* independent of *Her-2/neu* status (18, 33). Treatment with paclitaxel of tumors formed by inoculation of 231-E1A cells achieved 80% reduction of tumor weight in

comparison with untreated control 231-E1A tumors. Paclitaxel treatment of tumors formed by 231-Vect cells also achieved nearly 50% reduction of tumor weight. These results indicate that paclitaxel alone is sufficient to achieve a fifty-percent cytoreduction of tumor mass in comparison with mice treated with normal saline (NS) (Figure 2A). Comparing the percentage of tumor shrinkage in paclitaxel treated 231-Vect tumor group, paclitaxel treatment of 231-E1A tumor achieved a better tumor shrinkage than that of the paclitaxel treated 231-Vect animals, i.e., the 231-E1A group was more sensitive to Taxol ($P < 0.01$). Thus, expression of E1A also sensitized paclitaxel cytotoxic effect in established tumors *in vivo*.

To test if E1A was still expressed in tumor tissue in vivo, we then analyzed E1A expression in tumor tissue slides and used original 231-E1A stable cells prepared by cytospun as the positive control by immunohistochemistry. Modest to strong E1A staining was detected in more than ninety percent of 231-E1A stable cells cultured *in vitro* in the cytospun slides (Figure 2B). Whereas, less than half of the cells were positive for E1A staining in tumor tissue samples obtained from the original 231-E1A inoculated animals, and most of them only had modest or weak staining of E1A (Figure 2B). This result suggests that most of the cells with higher E1A-expressing were lost under the *in vivo* selection pressure during tumor development. None of the control 231-Vect stable cells or 231-Vect inoculated tumors had a positive staining for E1A (Figure 2B). These results also suggest that expression of E1A repressed tumor growth in vivo while loss of E1A expression may result in tumor development in 231-E1A stable cells.

E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis *in vivo*

To test if E1A-mediated sensitization to paclitaxel cytotoxicity *in vivo* is also through apoptotic cell death, we used a deoxynucleotide transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay to compare apoptotic index in paclitaxel-treated tumor tissue samples obtained from animals inoculated with 231-E1A cells versus those from animals inoculated with 231-Vect cells. In order to detect apoptotic cells *in vivo* following paclitaxel treatment, tumor-bearing animals were treated with paclitaxel (15mg/kg/animal) once by i.p. injection. Animals were sacrificed 24 hours after paclitaxel treatment and tumor tissue samples were then collected and sectioned for TUNEL assay. In tumor tissues obtained from 231-E1A-inoculated animals (n=3), a few TUNEL-positive cells were detected without treatment with paclitaxel, however, treatment with paclitaxel remarkably increased TUNEL-positive cells (from 9% to 18% positive cells). Treatment with paclitaxel also enhanced TUNEL-positive cells in tumor tissue samples obtained from 231-Vect-inoculated animals (n=3) (from 3% without paclitaxel to 7% with paclitaxel) (Figure 3 A and B). Statistic analysis showed that paclitaxel-treated 231-E1A tumor tissues had a significantly higher percentage of TUNEL-positive cells than did paclitaxel-treated 231-Vect tumor tissues ($p < 0.001$). Again, the result suggests that expression of E1A significantly enhanced paclitaxel cytotoxicity in tumor tissue *in vivo*. The relatively higher frequency of TUNEL-positive cells detected in tumor tissues obtained from 231-E1A-inoculated animals also suggests that the relatively small tumors formed in 231-E1A-inoculated animals in Figure 2A may in part due to the enhanced apoptosis in 231-E1A tumors.

Enhancement of paclitaxel-induced anti-tumor effect by systemic delivery of E1A gene in an orthotopic breast cancer model *in vivo*

To explore whether E1A could directly enhance paclitaxel-induced killing in a gene therapy setting *in vivo*, we designed systemic E1A gene therapy experiments in mice by intravenous (*i.v.*) injection of liposome encapsulated E1A gene via the mouse tail vein with or without combinaiton of paclitaxel chemotherapy. MDA-MB-231 cells were inoculated orthotopically into mouse m.f.p. and were allowed to grow until a palpable tumor was formed. When tumors were established, animals were regrouped and randomly assigned to each experimental group. We used SN liposome formulation as our gene-delivery system because of its relatively high efficiency *in vivo* (28). SN liposome (SN) or SN liposome encapsulated E1A gene was given twice a week for six weeks through mouse tail vein, while paclitaxel were given once a week by intra-peritoneal injection for six weeks. We started to monitor tumor size once a week when animals received the first time of each treatment and continued until six weeks later after each treatment were ended (total for 12 weeks). We then compared the mean tumor volume between each treatment group in order to assess the therapeutic effects of each treatment regime. Compared with control SN liposome alone, treatment with either systemic delivery of E1A gene or *i.p.* injection of paclitaxel repressed tumor growth during the 12-week observation period ($P < 0.05$, $P < 0.01$) (Fig. 4). Compared with treatment of systemic E1A gene alone or paclitaxel alone, a combination of E1A gene therapy and paclitaxel chemotherapy produced significantly enhanced therapeutic effect of paclitaxel and dramatically repressed tumor growth (both $P < 0.0001$) (Fig. 4). In addition, tumors were completely eradicated in four out of seven mice treated with combination therapy. Thus, combination of systemic E1A gene therapy with paclitaxel chemotherapy had a

synergistic effect in repressing tumor growth in the orthotopic breast cancer model *in vivo*.

Prolonged animal survival rate by E1A gene therapy in combination with paclitaxel chemotherapy.

In order to see if combination of systemic E1A gene therapy with paclitaxel chemotherapy could increase animal survival time, we extended our observation on animals after receiving the above treatments. The end point criteria include either tumor size reached 2 cm in diameter or animals are moribund. In animals that received treatment with liposome alone, most of the animals were died or sacrificed (because their tumors reached end point criteria) within three months after treatment. Although E1A-liposome alone did not dramatically repress tumor growth as that of paclitaxel alone did (Figure 4), treatment with systemic delivery of E1A gene achieved a comparable enhancement than that of the animals treated with paclitaxel alone in terms of prolongation of the animal survival times ($P < 0.05$) (Figure 5). Compared with survival rate of animals with treatment of SN liposome, treatment with either E1A gene or paclitaxel had a longer survival time (both $P < 0.05$). In nearly a year's close observation, we found that combination of systemic E1A gene therapy with paclitaxel chemotherapy achieved a significantly better outcome in animal's survival rates, compared with all the three single treatment regimes, including SN-liposome alone, liposome-E1A, or paclitaxel (all $P < 0.01$) (Fig. 5). In addition, three out of seven animals treated with combination therapy achieved over one year's tumor-free survival. Thus, combination of systemic E1A gene therapy with paclitaxel chemotherapy significantly enhanced

paclitaxel's antitumor effect and dramatically prolonged animal survival rates in the orthotopic breast cancer model *in vivo*.

DISSCUSION

In the current study, we showed that E1A mediated sensitization to the paclitaxel-induced apoptosis *in vitro* in low *Her-2/neu* expression breast cancer MCF-7 and MDA-MB-231 cells. We showed that expression of E1A also enhanced the therapeutic effect of paclitaxel in an orthotopic breast cancer model *in vivo*. In addition, we demonstrated that combination of a systemic E1A gene therapy using a surface-protected SN-liposome E1A complex formulation with paclitaxel chemotherapy significantly enhanced paclitaxel's antitumor effect and dramatically prolonged animal survival rates in an orthotopic breast cancer animal model. Our data showed that the combination of both E1A gene therapy and paclitaxel chemotherapy is much more efficient than either E1A gene therapy or paclitaxel alone in terms of repression of tumor growth *in vivo* and prolongation of animal survival rate. Unlike the trials on *Her-2/neu* overexpression tumors that used *Her-2/neu* expression as the marker, test on low *Her-2/neu* expression tumors may need a surrogate marker or markers in order to monitor if the therapy works in this subgroup of tumors. In the literature, previous studies on E1A-mediated sensitization to drug-induced apoptosis in different cell systems or cell free systems have identified a few other molecules that are also linked to E1A-mediated chemosensitization other than down-regulation of *Her-2/neu*, such as the pro-apoptotic protein Bax, Apaf-1, p19ARF, pro-caspase-2, -3, -7, -8, and –9, cell cycle inhibitor $p21^{Cip1/Waf-1}$, or an yet unidentified inhibitor that ordinary provides protection against cell death (23, 32, 34-41). Whether one

of these molecules or other unknown molecules could be used as a surrogate marker to monitor the therapeutic effect of this combination therapy in tumors with low *Her-2/neu* expression needs further assessment.

It is worthwhile mentioning the important clinical implications that the concentration of paclitaxel we tested in the *in vitro* study. Because the paclitaxel concentration that can kill cancer cells *in vitro* in the presence of E1A is clinically relevant. A plasma concentration of 5 μM to 10 μM paclitaxel can be achieved after bolus infusion of paclitaxel, but it rapidly falls to a level of several hundred nanomolar or less (42). Our experiments showed that 10 nM paclitaxel was sufficient to induce apoptosis *in vitro* in E1A-expressing cells but not in parental or vector control cells (Figure 1). This indicates that clinically relevant concentration (5~200 nM) of paclitaxel is sufficient to kill E1A-expressing cells, but parental cells require much higher dosage, which may be difficult to be achieved in a clinical setting (42).

We have previously shown that *Her-2/neu* overexpressing cells are resistant to paclitaxel-induced apoptosis (43, 44) and E1A, through downregulation of *Her-2/neu*, can sensitize cellular response to paclitaxel-induced apoptosis (22, 23, 43, 44). In those studies, we could not detect E1A-mediated chemosensitization in the low *Her-2/neu*-expressing cells (22). The major reason for this discrepancy was due to the paclitaxel concentrations tested. The *Her-2/neu*-overexpressing cancer cells are resistant to paclitaxel even at a dosage of 10.0 μM, in the presence of E1A, they became sensitive even at the dose of 1.0 μM of paclitaxel (22, 23). The low *Her-2/neu*-expressing cells, such as MDA-MB-231and MDA-MB-435, are much more sensitive to paclitaxel, even at paclitaxel concentration of 0.1 μM they are still sensitive (22). Therefore, in the previous

studies, the dose of paclitaxel was too high (1 μM) to detect E1A-mediated paclitaxel sensitization that became detectable in the present study (0.01 μM). Thus, the E1A gene therapy strategy may represent a novel and unique way to enhance the sensitivity of tumor cells to chemotherapy and the current study provides solid pre-clinical data that may help in developing further clinical trials using the combination of chemotherapy with systemic gene therapy.

Acknowledgement

This work was partially supported by the National Breast Cancer Foundation, Inc., RO1-CA58880 grant from the National Institutes of Health, and by grant DAMD17-01-1-0300 from the United States Department of Defense Army Breast Cancer Research Program (to Y. L.).

Legend to Figures

Figure 1. E1A–mediated sensitization to paclitaxel-induced apoptosis *in vitro*.

a: MTT assay. Percentage of viable cells after exposure to 0.1μM, 0.01 μM, and 0.001 μM of paclitaxel (Taxol) for 24 hr in MDA-MB-231 (231), vector-transfected cells (Vect), and E1A-expressing cells (E1A) detected using MTT assay. The number of viable cells without paclitaxel treatment was defined as 100%. The insert shows a cleaved PARP p89 fragment (ΔPARP) that was detected using a rabbit polyclonal antibody against cleaved PARP. Lanes 1, 5, and 9 in the insert represent cleaved PARP products from cells without paclitaxel treatment.

b: Percentage of viable cells after exposure to paclitaxel in MCF-7 cells, MCF-7/E1A-expressing cells (MCF-7-E1A), and a revertant of E1A-expressing clone (E1A-R) detected using MTT assay. The insert shows ΔPARP, and lanes 1, 5, and 9 in the insert represent cleaved PARP products from cells without paclitaxel treamtment.

c: FACS analysis of sub-G1–phase apoptotic cells with or without exposure to 0.01 μM paclitaxel for 24 hours. The symbols used are the same as those in panels A and B. DMSO, dimethyl sulfoxide.

Figure 2. E1A enhanced paclitaxel anti-tumor effect in nude mice *in vivo*.

a: Tumor volume of animals inoculated with MDA-MB-231-Vect (231-Vect) or MDA-MB-231-E1A (231-E1A) stable cells with or without treatment with paclitaxel. At least 5 animals were included in each group.

b: Immunohistochemical analysis of E1A expression in 231-Vect (a, b) and 231-E1A (c, d) cells growing in tissue culture *in vitro* (a and c, respectively) and in tumor tissue sample *in vivo* (b and d, respectively) after cells were inoculated into animal.

Figure 3. TUNEL assay of apoptotic cells in tumor tissue *in vivo*.

a: TUNEl labelling of apoptotic cells in tumor tissue samples obtained from 231-Vect or 231-E1A inoculated animals with (b and d) or without (a and c) treatment with paclitaxel.

b: The percentage of TUNEL-positive cells in the tumor tissues samples obtained from 231-Vect and 231-E1A inoculated animals (each n=3) with or without treatment of paclitaxel.

Figure 4. Tumor volume of animals during or after treatment with systemic delivery of liposome-E1A alone with or without i.p. injection of paclitaxel. Treatment groups included SN-liposome vehicle (✳), SN-liposome-E1A (▲), paclitaxel alone (O), or paclitaxel plus SN-E1A (■). At least 7 animals were included in each group.

Figure 5. Survival curve of animals after treatment with systemic delivery of SN liposome alone (✳), i.v. liposome-E1A alone (▲), i.p. injection of paclitaxel (O), or combination of systemic liposome E1A and i.p. injection of paclitaxel (■).

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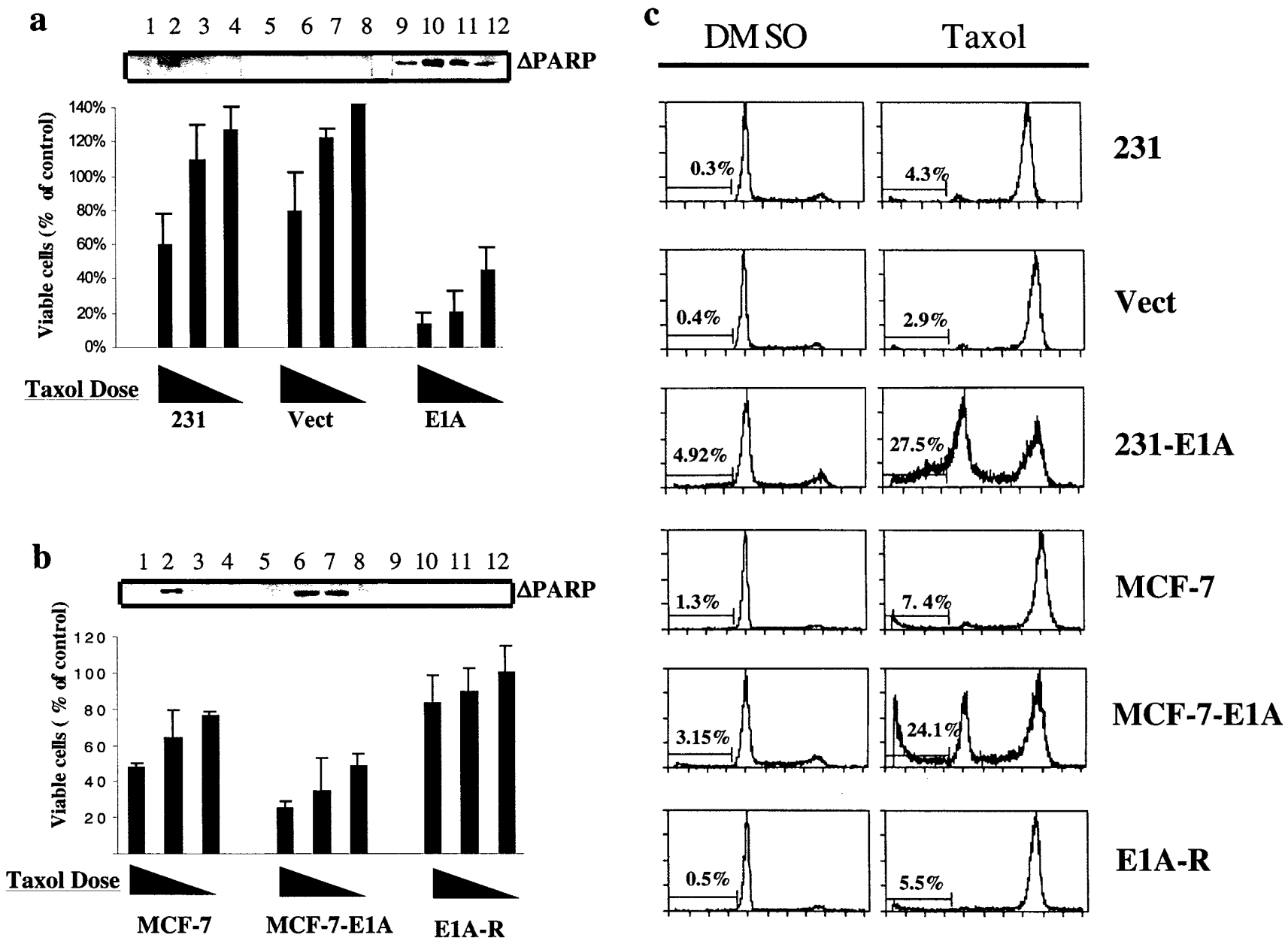
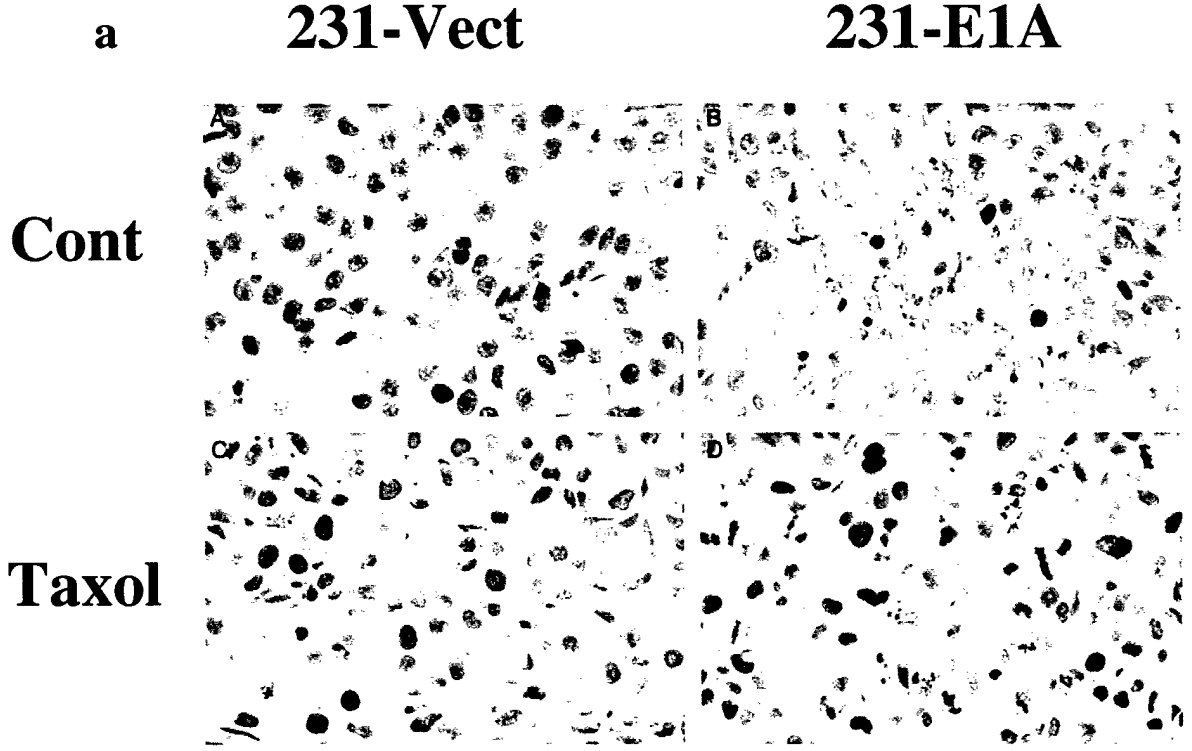

a
1 2 3 4 5 6 7 8 9 10 11 12
ΔPARP
Viable cells (% of control)
140%
120%
100%
80%
60%
40%
20%
0%
Taxol Dose
231
Vect
E1A
b
1 2 3 4 5 6 7 8 9 10 11 12
ΔPARP
Viable cells (% of control)
120
100
80
60
40
20
Taxol Dose
MCF-7
MCF-7-E1A
E1A-R
c
DMSO
Taxol
0.3%
4.3%
231
0.4%
2.9%
Vect
4.92%
27.5%
231-E1A
1.3%
7. 4%
MCF-7
3.15%
24.1%
MCF-7-E1A
0.5%
5.5%
E1A-R

Figure 1

Figure 2

a
Tumor Volume (mm³)
0
0.2
0.4
0.6
0.8
231-Vect (cont.)
231-Vect (Taxol)
231-EIA (cont.)
231-EIA (Taxol)
b
Cell line (in vitro)
Tumor tissue
231-Vect
231-E1A
A
B
C
D

a


231-Vect
231-E1A
Cont
Taxol
A
B
C
D

b

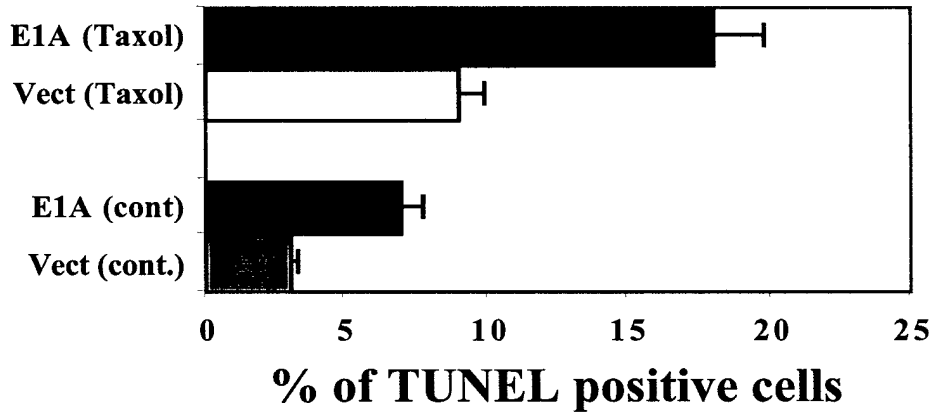

E1A (Taxol)
Vect (Taxol)
E1A (cont)
Vect (cont.)
0
5
10
15
20
25
% of TUNEL positive cells

Figure 3

Figure 4

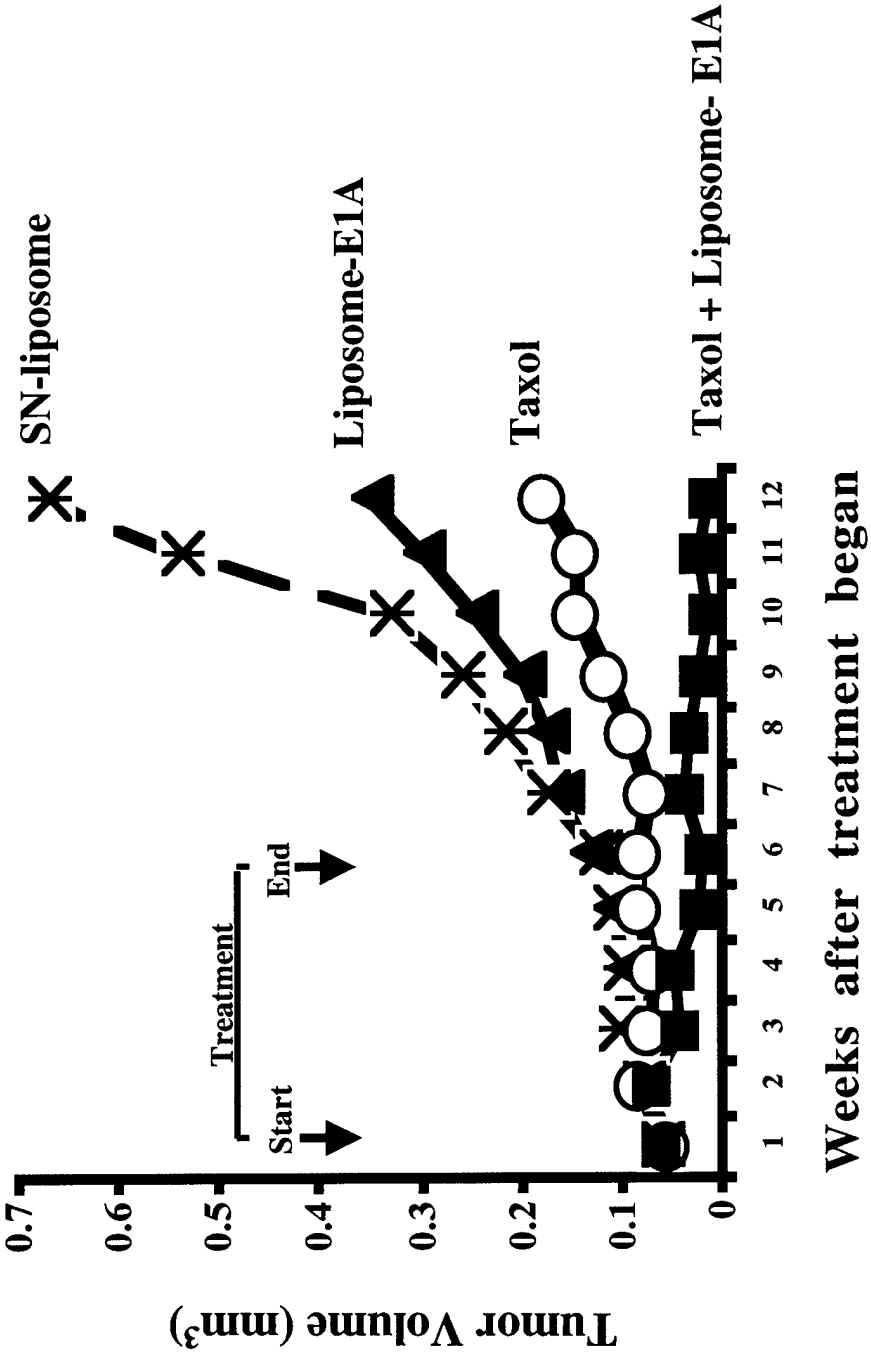
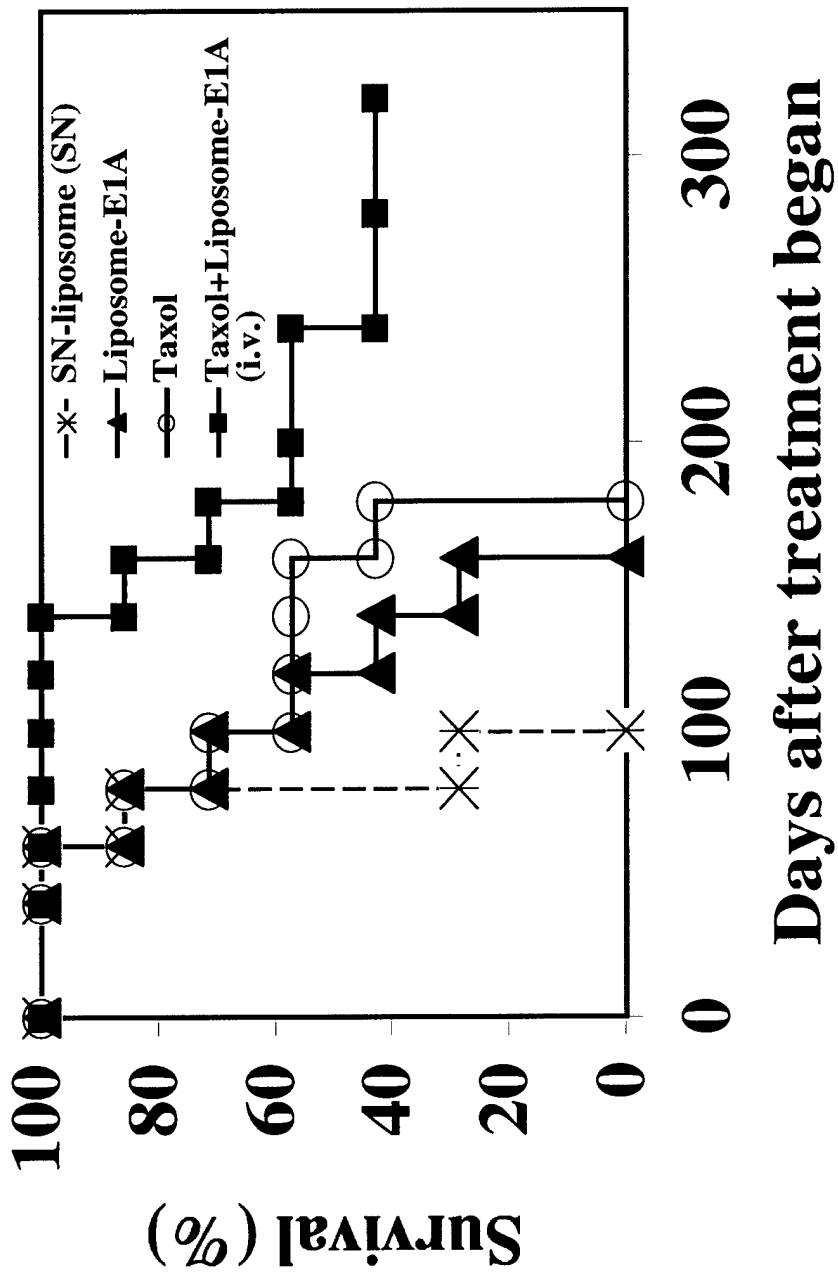

SN-liposome
Liposome-E1A
Taxol
Taxol + Liposome- E1A
Treatment
Start
End
Tumor Volume (mm3)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12
Weeks after treatment began

Figure 5


SN-liposome (SN)
Liposome-E1A
Taxol
Taxol+Liposome-E1A (i.v.)
Survival (%)
100
80
60
40
20
0
0
100
200
300
Days after treatment began

A new role of protein phosphotase 2A in adenoviral E1A protein mediated sensitization to anti-cancer drug–induced apoptosis in human breast cancer cells.

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Grant support: This work was partially supported by the National Breast Cancer Foundation, Inc., RO1-CA58880 grant, the SPORE grant for ovarian cancer research, CA83639 (to M.-C. H.) and Cancer Center Supporting Grant CA16772 from the National Institutes of Health and by grant DAMD17-01-1-0300 from the United States Department of Defense Army Breast Cancer Research Program (to Y. L.).

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Running title: PP2A in E1A-mediated chemosensitization in breast cancer.

Key words: PP2A, Akt, Adenovirus E1A, Chemosensitization, and breast cancer.

Abstract

The adenoviral type 5 E1A protein has been shown to induce sensitization to different categories of anticancer drug-induced apoptosis, partly by downregulation of the activity of a critical oncogenic kinase Akt in both normal fibroblasts and epithelial breast cancer cells (Liao Y & Hung MC, MCB, 2003; Viniegra JG, et al. Oncogene, 2002). Currently, the adenoviral E1A gene is being tested as an anti-tumor gene in multiple clinical trials. However, molecular mechanisms underlying E1A-mediated chemosensitization and downregulation of Akt activity are still not completely defined. Here, we show that E1A by upregulation of the catalytic subunit of PP2A (PP2A/C) enhanced the activity of PP2A, which results in repression of Akt activation in E1A expressing cells. In addition, activation of PP2A/C is required for E1A–mediated sensitization to drug–induced apoptosis, since blocking PP2A/C expression using a specific small interfering RNA (siRNA) against PP2A/C reduced drug sensitivity in E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, also abolished E1A's ability to upregulate PP2A/C. Thus, the upregulation of PP2A may represent a novel mechanism for E1A-mediated sensitization to anti-cancer drug-induced apoptosis.

Reversible phosphorylation of proteins by protein kinases and phosphatases is a key regulatory mechanism in the control of multiple cellular processes, ranging from cell proliferation and survival to cell death. Many oncogenes identified today are protein kinases, since their kinase activities are finely regulated by respective protein phosphatases, it is important to understand how the respective protein phosphatases are involved in the regulation of these cellular processes (1). So far, alterations or mutations of very few phosphatases have been implicated in the development of tumors and only the dual-specific protein phosphatase PTEN exhibits many characteristics of a typical tumor suppressor (2, 3). A tumor suppressive function of protein phosphatase 2A (PP2A) has been proposed, as a deletion mutation of the regulatory subunit of PP2A was found in primary human breast, colon, and lung tumors and melanoma (2) and inactivation of PP2A by the small t antigen of the DNA tumor virus simian virus 40 (SV40) was also found to be involved in viral-induced cell transformation (4). The core enzyme of PP2A is a dimer, consisting of a catalytic subunit (PP2A/C) and a regulatory or structural A subunit (PP2A/A). A third regulatory B subunit (PP2A/B), which determines substrate specificity, can be associated with this core structure (1, 2). Recent evidence indicates that PP2A forms stable complexes with protein kinase signaling molecules, indicating that it plays a central, regulatory role in signal transduction mediated by reversible protein phosphorylation (1). While the role of PP2A in the regulation of apoptosis is not clear, result from a gene knockout study of PP2A/C implies that it may play a critical role in regulation of apoptotic signaling (5). In supporting of this notion, several groups reported that PP2A through the dephosphorylation of a key oncogenic survival factor Akt

participated in the regulation of apoptosis induced by ceramide, mistletoe lectin, and 4-hydroxynonenal, an aldehyde product of membrane lipid peroxidation (6-8).

Recently, we and other groups have shown that repression of Akt activation by adenoviral E1A contributed to E1A-mediated sensitization to anticancer drug-induced apoptosis in both normal fibroblast and epithelial breast cancer cells (9-11). However, the mechanism involved in E1A-mediated repression of Akt activity is still not known. Here, we show that the PP2A phopshatase activity is enhanced in E1A-expressing cells through E1A-mediated upregulation of PP2A/C expression, which results in repression of Akt activation. We demonstrated that PP2A is involved in regulation of apoptosis and that activation of PP2A/C is also required for E1A–mediated sensitization to anti-cancer drug–induced apoptosis in E1A expressing breast cancer cells.

Materials and Methods

Cell lines. The stable E1A-expressing cell lines and domain deletion mutants were established as described previously (10).

Preparation of cell lysates, western blot analysis, and antibodies. Preparation of cell lysates and western blot analysis were performed according to standard protocols as previously discribed (10). Rabbit anti-human PP2A/A and PP2A/C were purchased from CalBiochem (La Jolla, CA). Information of other antibodies used were previously discribed (10).

Serine/threonine phosphatase assay. A nonradioactive serine/threonine phosphatase assay system was purchased from Promega Corporation (Madison, WI). PP2A phosphatase activity was measured according to the manufacturer's protocol.

Immunoprecipitation, PP2A treatment, and Akt dephosphorylation assay. To measure endogenous Akt dephosphorylation by exogenous purified recombinant human PP2A (rhPP2A) (Upstate Biotechnnology, NY), cells were stimulated using 10 μM insulin for 15 min before harvesting. To measure exogenous Akt dephosphorylation, HA-tagged Akt was transiently transfected into 293T cells. Cells were lysed, immunoprecipitated with anti-Akt or anti-HA antibodies. Aliquots of the Akt immunoprecipitates were incubated with various doses of rhPP2A at 30°C for 30 min, after which the reaction was terminated through the addition of 6X SDS loading buffer and resolved using 10% SDS-PAGE. Phosphorylated and total Akt and HA-tag were measured using anti-phospho-Akt (Thr308-p) and total Akt and anti-HA tagged antibody, respectively.

Synthesis and application of siRNA. Double stranded siRNAs were produced *in vitro* using chemically synthesized DNA oligonucleotide templates (Sigma, St. Louis, WA) and the T7-MEGAshortscipt™ kit (Ambion Inc., Austin, TX) according to the T7 siRNA protocol described by Paddison et al. (12). The specific primer sequences for PP2A/C are:

A: 5' CCG AGT CCC AGG TCA AGA G CC TAT AGT GAG TCG TAT TAC 3'

B: 5' GAG GCT CTT GAC CTG GGA C CC TAT AGT GAG TCG TAT TAC 3'

The non-specific scrambled control primer sequences are:

A: 5' ATG GAG AGC AGG TCA AAC T CC TAT AGT GAG TCG TAT TAC 3'

B: 5' TTG GAG TTT GAC CTG CTC T CC TAT AGT GAG TCG TAT TAC 3'

Statistical analysis. Statistical analysis was performed with a two-tailed Student's *t* test, and $P < 0.05$ was considered statistically significant.

Results and Discussion

PP2A activity is enhanced, which is correlated with elevated expression of the catalytic subunit of PP2A (PP2A/C) in stable E1A-expressing cells. Phosphorylation of protein kinases are tightly regulated by related protein phosphatases and two phosphatases, PTEN and PP2A, have been shown to repress Akt activation through dephosphorylation (3, 6-8). In order to identify whether protein phosphatases were involved in E1A-mediated downregulation of Akt activation, we measured the alteration of protein phosphatases, such as PTEN, PP2A, in stable E1A-expressing cells versus that in vector control cells. We did not detect any change in PTEN expression in stable E1A-expressing cells versus control cells (data not show). Also, there was no change in the expression level of the PP2A regulatory A subunit PP2A/A, however, we did detect elevated expression of the catalytic subunit of PP2A (PP2A/C) in multiple stable E1A expressing cells (Figure 1A). Thus, we further tested if PP2A activity was increased in the E1A-expressing cells by using a specific PP2A phosphatase assay. We observed that the PP2A activity was enhanced in E1A-expressing MDA-MB-231 cells (231-E1A) in a dose (protein and substrate concentration) dependent manner compared to that of the vector control cells (231-Vect)(both $P< 0.01$, Fig. 1B). The above results suggest that E1A by upregulating PP2A/C expression enhance the activity of PP2A.

PP2A is involved in the regulation of Akt and p38 activities. Next, we asked if enhanced PP2A activity in E1A-expressing stable cells contributed to E1A-mediated repression of Akt activation. First, we verified whether Akt could be dephosphorylated by

recombinant human PP2A (rhPP2A), which contains both the catalytic and the regulatory A subunits. Dephosphorylation of endogenous Akt occurred in a rhPP2A dose-dependent manner (Fig. 1C). Similar to the dephosphorylation of endogenous Akt, the HA-tagged, exogenous Akt was also dephosphorylated by rhPP2A in a dose-dependent manner *in vitro* (data not show). To test if dephosphorylation of Akt is dependent on PP2A activity, we used the specific PP2A inhibitor okadaic acid (OA) to block PP2A activity and measured Akt phosphorylation in the presence of rhPP2A. We found that dephosphorylation of both endogenous and exogenous Akt was completely abolished in the presence of 1nM PP2A inhibitor OA (Fig. 1C and data not show). This result suggests that dephosphorylation of Akt is mediated by PP2A and dependent on PP2A phosphatase activity *in vitro*.

To test if dephosphorylation of Akt by PP2A also occurs in cells in vivo, stable E1A expressing 231-E1A were treated with OA and the Akt phosphorylation level was monitored for 24 hrs. In the presence of OA, the Akt phosphorylation level was significantly increased at 12 hrs and the increment was subsequently reduced at 24 hrs, presumably due to the limited half-life of OA (Fig. 1D). Previously, we and other groups have shown that activation of Akt results in inactivation of p38 (10, 13), therefore we also measured p38 phosphorylation before and after exposure with OA. In consistent to the previous studies, phosphorylation of p38 was detected before exposure to OA (0 hr) and was repressed at 12 hrs when Akt phosphorylation was increased. Additionally, p38 phosphorylation was recovered at 24 hrs when accumulation of Akt phosphorylation was reduced (Fig. 1D). Both total Akt and total p38 protein levels had no change throughout exposure with OA (Fig. 1D). This result indicates that both Akt and p38 phosphorylation can also be regulated by PP2A *in vivo*.

Upregulation of PP2A/C is required for E1A–mediated chemosensitization. It has been reported that E1A could sensitize anticancer drug-induced apoptosis through downregulation of Akt activation (9, 10), we asked whether E1A-induced upregulation of PP2A/C might play a role in E1A-mediated chemosensitization. To test this, we measured PP2A/C expression during anticancer drug-induced apoptosis in both 231-Vect cells and 231-E1A cells. We used PARP cleavage as a marker of apoptosis and Bcl-2 phosphorylation as an indication of the pharmaceutical effect of the anti-microtubule drug paclitaxel (14). The protein level of PP2A/C was further increased and was correlated with reduced Akt phosphorylation, increased p38 phosphorylation and Bcl-2 phosphorylation, and increased PARP cleavage after exposure to paclitaxel in the 231-E1A cells, suggesting increased PP2A/C is correlated with drug-induced apoptosis in E1A-expressing cells (Fig. 2A). In addition, to test which apoptotic pathway is involved in E1A-mediated sensitization to paclitaxel-induced apoptosis, we measured the expression of caspase–3, –8, –9, and cytosolic fraction of cytochrome c (15). We observed that paclitaxel-induced PP2A/C expression and PARP cleavage in 231-E1A cells correlated with cytochrome c release and activation of procaspase–8, –9, and –3. This result suggests that both intrinsic (represented by cytochrome c release and caspase-9 cleavage) and extrinsic (represented by activation of procaspase–8) apoptotic pathways may be involved in E1A-mediated sensitization to paclitaxel-induced apoptosis (15). PARP cleavage and increased PP2A/C was also observed in the 231-Vect cells after treatment with paclitaxel, but to a much lesser extent, it implied that PP2A/C may be required for drug-induced apoptosis in the absence of E1A.

To test if the above observation can also be applied to E1A-mediated chemosensitization in other cell lines, additional two pairs of E1A-expressing stable cell lines and the vector DNA transfected controls were tested. We observed notably higher levels of PP2A/C, reduced Akt phosphorylation, enhanced p38 phosphorylation, and cleaved PARP fragment in E1A-expressing cells after exposure to paclitaxel compared to that of the corresponding vector controls. Again, enhanced expression of PP2A/C and cleaved PARP fragment in E1A-expressing cells after exposure to paclitaxel correlated with the activation of procaspase–3, –8, and –9 (Fig. 2B). Whereas the expression level of the regulatory subunit of PP2A/A was not significantly altered in E1A-expressing cells versus control cells (Fig. 2B). These data suggest that elevated expression of PP2A/C in E1A-expressing cells is involved in E1A–mediated sensitization to drug-induced apoptosis.

To further test if upregulation of PP2A/C expression by E1A is required for E1A–mediated sensitization to apoptosis, we used a double-strand small interfering RNA (siRNA) against PP2A/C as a tool to knockdown PP2A/C expression in E1A–expressing cells. First, we did a dose escalation study of siRNA on PP2A/C protein expression, we found 5.0 μg siRNA was sufficient to repress PP2A/C expression (~60% reduction in PP2A/C protein expression) in 231-E1A cells (Fig. 2C). We then tested if repression of PP2A/C expression would also inhibit drug-induced apoptosis in E1A–expressing cells. We exposed 231-E1A cells to either a specific siRNA against PP2A/C or a scrambled, non-specific control siRNA in the presence or absence of paclitaxel for 24 hours. We then measured the expression levels of cleaved PARP and PP2A/C and counted events of nuclear fragmentation under microscopy as an alternative measure for the occurrence of

apoptotic cells. We detected a 2-fold increase in the expression level of PP2A/C and 2.5-fold increase of cleaved PARP in the presence of paclitaxel as compared with cells treated with control siRNA alone without paclitaxel (Fig. 2D, lanes 1 versus lane 2). However, when compared with cells in the presence of control siRNA, cells treated with PP2A/C specific siRNA had reduced both PP2A/C expression and cleaved PARP (0.5-fold of PP2A/C and 0.2-fold cleaved PARP proteins) (Fig. 2D, lane 1 versus lane 3). In the presence of both PP2A/C specific siRNA and paclitaxel, the increment of PP2A/C expression is minimal (0.7-fold that of control siRNA alone) and the cleaved PARP protein is comparable with control siRNA alone (0.9-fold) in the absence of paclitaxel (Fig. 2D, lane 1 versus lanes 4). In consistent with the above results, we also observed that when PP2A/C expression was blocked by specific siRNA, Akt phosphorylation was elevated while p38 phsophorylation was reduced (Fig. 2D). Corresponding with the expression of PP2A/C and cleaved PARP proteins, the rate of DNA fragmentation in control siRNA treated cells is about 9 % (18/200) in the absence of paclitaxel and 21% (42/200) in the presence of paclitaxel. Whereas, in PP2A/C specific siRNA treated cells, the rate of DNA fragmentation is about 4% (8/200) in the absence of paclitaxel and 12% (24/200) in the presence of drug (Fig. 2E).

Taken together, the above results suggest that upregulation of PP2A/C is required for E1A–mediated sensitization to drug–induced apoptosis.

A PP2A phosphatase activity is also involved in apoptosis induced by different apoptotic stimuli, such as TNF-α. The above results established that by regulation of the Akt activation, PP2A played a role in adenoviral E1A-mediated sensitization to anti-cancer drug-induced apoptosis by repressing Akt activation and upregulation of p38

activation. In the previous report, we have shown that repression of Akt activation and upregulation of p38 activation contributed to different apoptotic stimuli-induced apoptosis, such as exposure to tumor necrosis factor (TNF)-α (10). To further test if PP2A plays a general role in the regulation of apoptosis by different apoptotic stimuli in the absence of E1A, we treated MDA-MB-231 cells with TNF-α at a dose that could induce apoptosis (10). PP2A phosphatase activity was measured after treatment. As experimental controls, we also treated MDA-MB-231 cells with insulin-like growth factor-1 (IGF-1), which is known to transiently stimulate the PI3K-Akt pathway, and MEK inhibitor PD58098, which is not supposed to affect Akt or p38 phosphorylation. We again used the cleaved PARP fragment (p89PARP) as a marker for apoptosis. As expected, we detected cleaved PARP in MDA-MB-231 cells after treatment with TNF-α for 24 hrs, correspondingly, we detected increased p38 phosphorylation and reduced Akt phosphorylation. Interestingly, we also observed increased PP2A phosphatase activity after treatment with TNF-α (Fig. 3). However, treatment with the MEK inhibitor PD58098 did not induce detectable PARP cleavage, alteration of Akt and p38 phosphorylation, or alteration of PP2A activity. Although IGF-1 did not dramatically affect Akt or p38 phosphorylation at the 24 hrs time point, it slightly reduced PP2A activity (Fig. 3). These results suggest that PP2A may also be involved in TNF-α induced PARP cleavage and apoptosis. In addition, when Akt activity was blocked by PI3K inhibitor Wortmannin, PP2A activity was increased, which correlated with decreased Akt phosphorylation, increased p38 phosphorylation, and PARP cleavage (Fig. 3). Additionally, blocking Akt activation using another PI3K inhibitor, LY249002, also

increased PP2A activity (data not shown). Thus, the PP2A activity is also involved in apoptosis induced by the blockade of PI3K-Akt pathway.

A conserved domain of E1A is required for upregulation of PP2A/C and sensitization to drug-induced apoptosis. Since PP2A/C is required for sensitization to drug–induced apoptosis, we then asked whether a deletion mutation of any conserved domain (CR) of E1A, which is required for E1A to sensitize cells to drug-induced apoptosis, also disrupts the ability of E1A to upregulate PP2A/C expression. In our previous report, we had mapped that among the three conserved domains of E1A, CR2 is associated with E1A–mediated sensitization to drug-induced apoptosis (10). Therefore, we utilized the same set of wild type (WT) E1A or conserved domain deletion mutant stable cells (ΔCR1, ΔCR2, and ΔCR3) established in MDA-MB-231 cells to test if the same deletion mutation would affect E1A–mediated upregulation of PP2A/C (Fig. 4A). As expected, we found that deletion mutation of the CR2 domain impaired E1A's ability to upregulate PP2A/C expression while the CR1 or CR3 domain mutant only slightly affected E1A–mediated upregulation of PP2A/C in the presence of paclitaxel (Fig. 4B). Thus, this result further supports that E1A–mediated chemosensitization requires upregulation of PP2A/C.

Taken together, in the current study, we showed that E1A-mediated sensitization to drug-induced apoptosis involves activation of PP2A through upregulation of PP2A/C expression, which results in activation of p38 and repression of Akt. In addition, activation of PP2A/C is required for E1A–mediated sensitization to drug–induced apoptosis, as blocking PP2A/C expression by a siRNA against PP2A/C reduced drug sensitivity of E1A-expressing cells. Deletion mutation of the conserved domain of E1A, which is required for E1A-mediated sensitization to drug-induced apoptosis, abolished

E1A's ability to upregulate PP2A/C (Fig. 4B) and downregulate Akt activation (10). Thus, by repressing Akt activation through PP2A, E1A upregulates p38 and facilitates cytochrome c release from mitochondria (Fig. 2A), which in turn, contributes to E1A-mediated sensitization to drug-induced apoptosis.

Acknowledgments

We thank Dr. Stephanie Miller for her reading and editing the manuscript.

Figure Legends

Fig. 1. PP2A activity is enhanced in stable E1A-expressing cells through upregulation of PP2A/C. *A*, Protein expression of PP2A/A, PP2A/C, E1A, and actin in stable E1A-expressing cells (E) and corresponding vector controls (V). *B*, PP2A activity was measured using a protein phosphatase assay kit. The substrate phospho-peptide concentrations were 20, 40, 100, 200, and 400 μM, respectively. Protein concentrations include 0.1, 1.0, and 10 μg, respectively. **V**, 231-Vector. **E**, 231-E1A. Results shown here are from free independent experiments. *C*, Dephosphorylation of Akt by purified hPP2A (1 mU = 1 x 10^{-3} U) and inhibition of by okadaic acid (OA; 1 nM) in vitro. Aliquots of immunoprecipitated endogenous Akt was incubated with purified human PP2A enzyme (hPP2A), relative phosphatase activities were measured by anti-phospho-Akt (T308) antibody. Total Akt was used as a loading control. *D*, Blocking PP2A activity by exposing 231-E1A cells to okadaic acid (10 nM) increased Akt phosphorylation and inhibited p38 phosphorylation.

Fig. 2. Upregulation of PP2A/C is required for E1A-mediated chemosensitization. *A*, PARP cleavage and PP2A/C activation in 231-Vect and 231-E1A cells after treatment with paclitaxel. Cyt. c: cytochrome c. *B*, Western blot analysis of PP2A and the catalytic subunit PP2A/C in Vect (**V**) or E1A (**E**) transfected MCF-7 and MDA-MB-453 cells after exposure to 0.01 μM and 1.0 μM of paclitaxel, respectively. *C*, A dose-finding test of specific PP2A/C siRNA on PP2A/C expression in 231-E1A cells. Relative intensity of PP2A/C was shown in the bottom. *D*, Cells were transfected with siRNA using JetSI™ cationic transfection reagent (Obiogene, Inc., Carlsbad, CA) for 16 hrs and replaced with

fresh medium before addition of 0.01 μM of paclitaxel and were incubated for another 24 hrs before harvesting. Relative intensities of PARP and PP2A/C in specific siRNA (PP2A/C) and non-specific control siRNA (Control) protected cells were shown in the bottom of each band. *E*, For nuclear fragmentation analysis, cells were grown in Lab-Tek® Chamber Slides™ (Nunc, Inc., Naperville, IL) and were treated by the same procedure as described above. Cells were then washed with PBS twice and fixed with 70% alcohol and stained with Hoechst 33342 (0.5 μg/ml, Sigma, St. Louis, Missouri). Events of apoptotic nuclei were counted under a fluorescence microscope and the mean values in every 200 cells in each field were plotted.

Fig. 3. Enhanced PP2A phosphatase activity in TNF-α induced apoptosis. PP2A phosphatase activity and Western blot analysis of the expression of phospho-Akt, phospho-p38, and PARP cleavage in MDA-MB-231 cells with treatment of IGF-1 (50 ng/ml), TNF-α (50 ng/ml), the PI3K inhibitor Wortmannin (WORT; 0.5 μM), and the MEK1/2 inhibitor PD98058 (PD; 20 μM).

Fig. 4. Conserved domains of E1A required for upregulation of PP2A/C. *A*, A domain structure and map for deletion mutation of CR1 and CR2. *B*, Western blot analysis of PP2A/C in vector-transfected (V), or wild type E1A (WT) and mutant E1A (ΔCR1, ΔCR2, or ΔCR3). Actin was used as a loading control.

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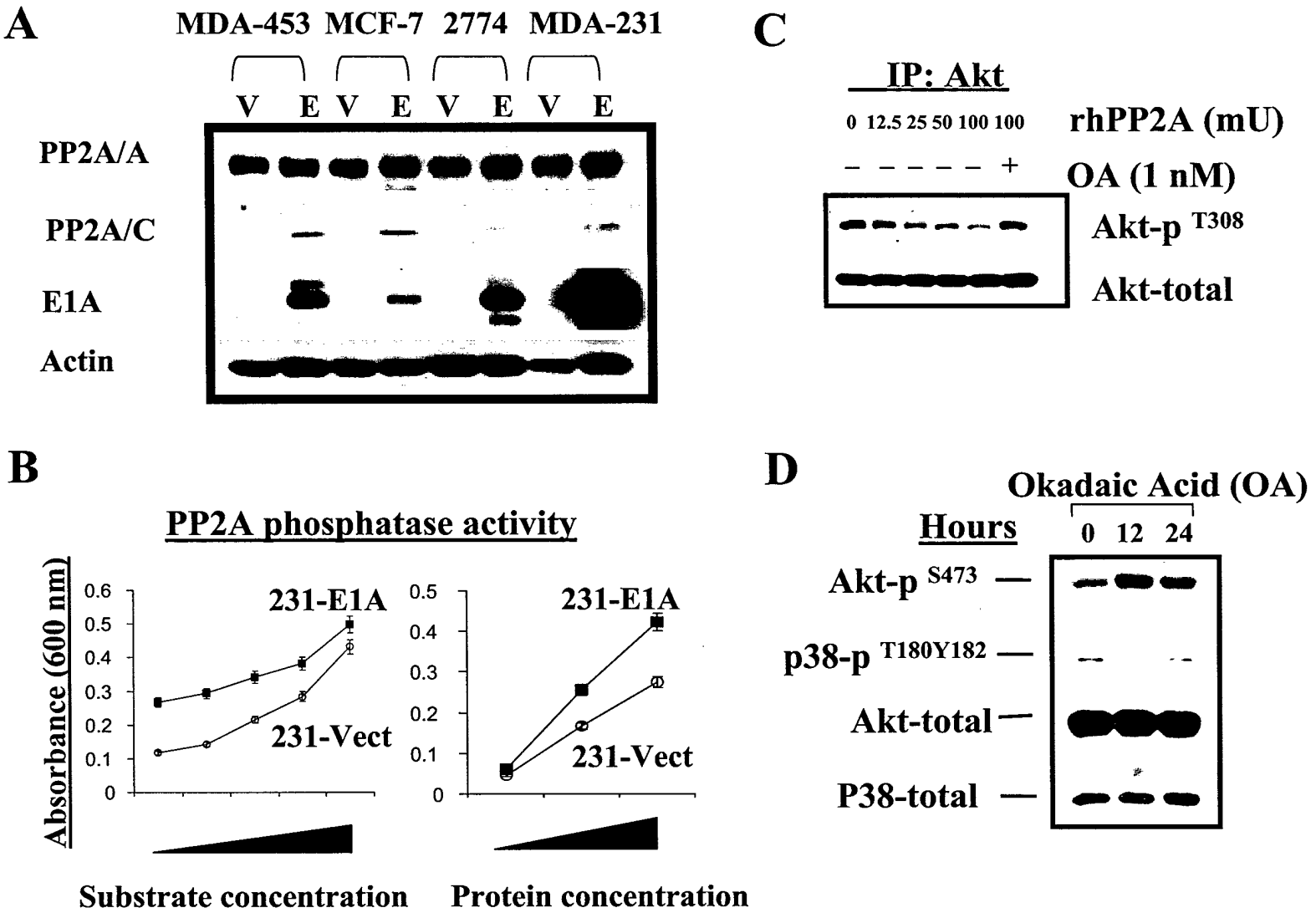
A
MDA-453 MCF-7 2774 MDA-231
V E V E V E V E
PP2A/A
PP2A/C
E1A
Actin
B
PP2A phosphatase activity
Absorbance (600 nm)
231-E1A
231-Vect
Substrate concentration
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C
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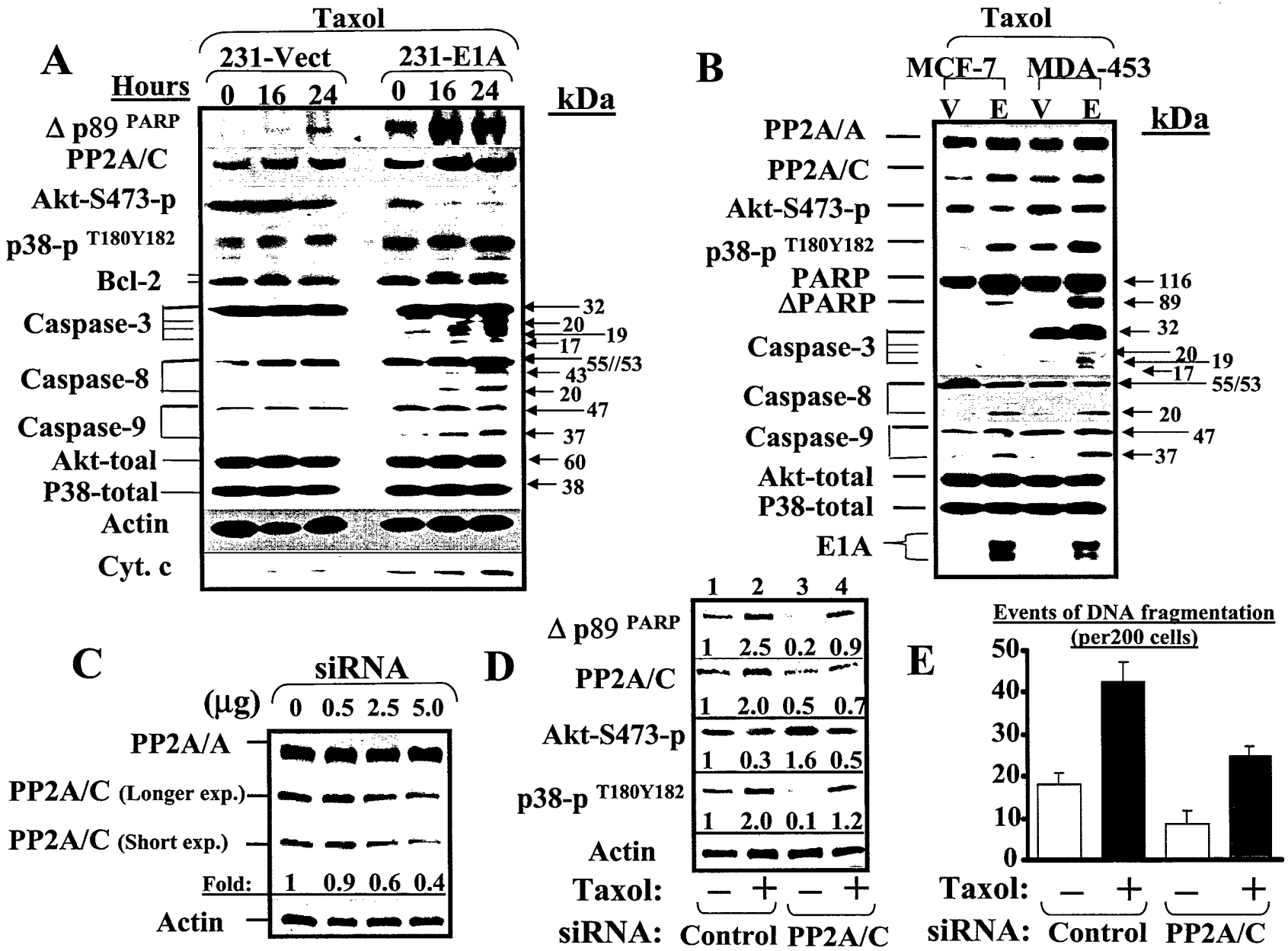
Akt-p S473
p38-p T180Y182
Akt-total
P38-total

Figure 1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PP2A/C (Longer exp.)
PP2A/C (Short exp.)
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Actin
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1 2 3 4
Δ p89 PARP
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PP2A/C
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Akt-S473-p
1 0.3 1.6 0.5
p38-p T180Y182
1 2.0 0.1 1.2
Actin
Taxol: – + – +
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Events of DNA fragmentation
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Taxol: – + – +
siRNA: Control PP2A/C

Figure 2

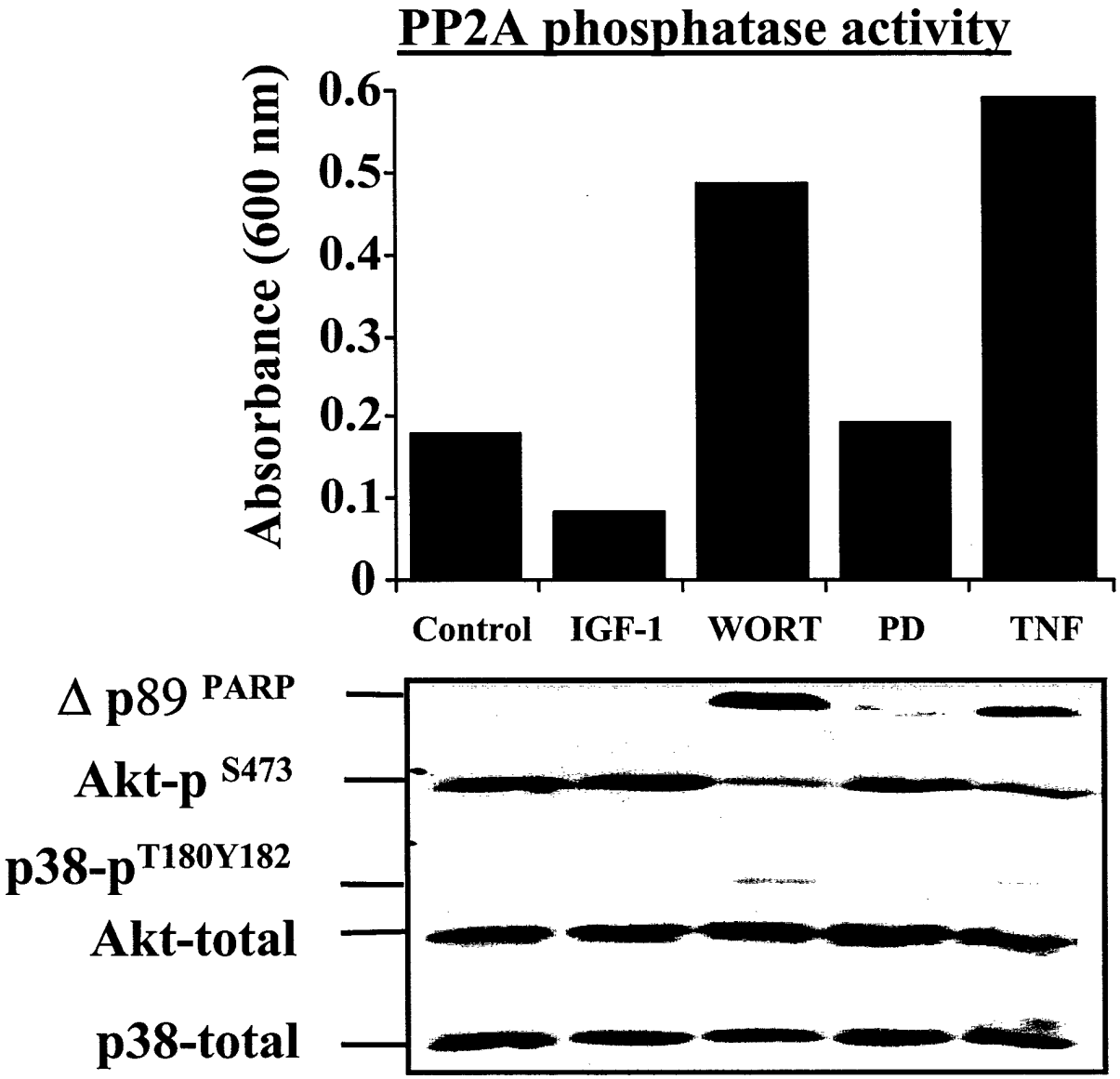
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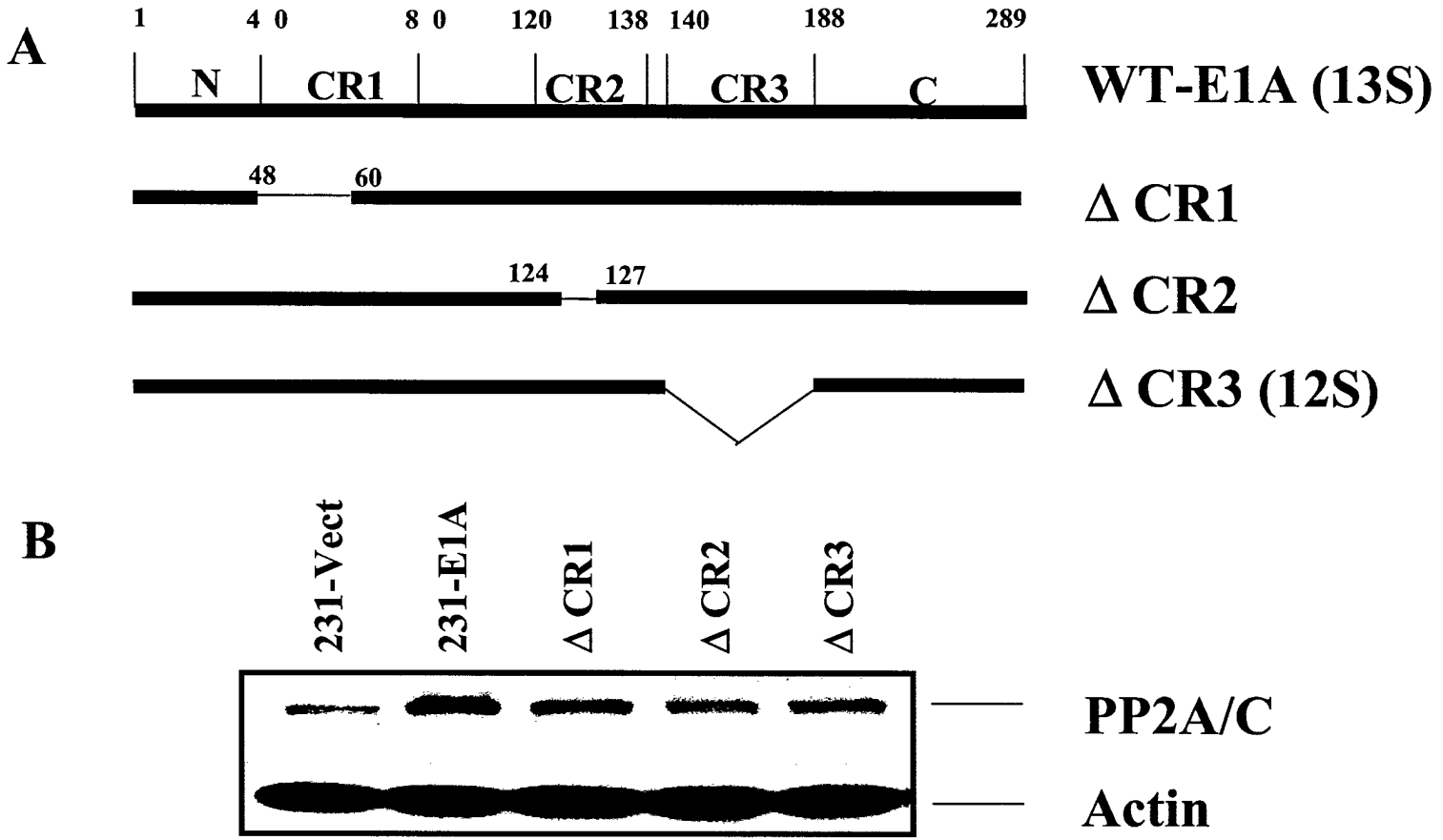
Figure 3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Δ CR1
124
127
Δ CR2
Δ CR3 (12S)
B
231-Vect
231-E1A
Δ CR1
Δ CR2
Δ CR3
PP2A/C
Actin

Figure 4

Phosphorylation/Cytoplasmic Localization of p21$^{Cip1/WAF1}$ Is Associated with HER2/*neu* Overexpression and Provides a Novel Combination Predictor for Poor Prognosis in Breast Cancer Patients

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ABSTRACT

Purpose: **The diversity of biological functions makes p21$^{Cip1/WAF1}$ (p21) a controversial marker in predicting the prognosis of breast cancer patients. Recent laboratory studies revealed that the regulation of p21 function could be related to different subcellular localizations of p21 by Akt-induced phosphorylation at threonine 145 in HER2/*neu*-overexpressing breast cancer cells. The purpose of this study was to verify these findings in clinical settings.**

Experimental Design: **The expression status of the key biological markers in the HER2/*neu*-Akt-p21 pathway in 130 breast cancer specimens was evaluated by immunohistochemical staining and correlated with patients' clinical parameters and survival. In addition, an antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] was also used for better validation of these findings.**

Results: **Cytoplasmic localization of p21 is highly correlated with overexpression of phospho-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) are associated with high expression of HER2/*neu* and phospho-Akt. Cytoplasmic localization of p21 and overexpression of phospho-p21 (T145), HER2/*neu*, and phospho-Akt are all associated with worse overall survival. Multivariate analysis of the Cox proportional hazard regression model revealed that cytoplasmic p21 and overexpression of HER2/*neu* are independently associated with increased risk of death. Combining these two factors stratified patients' survival into four distinct groups, with a 5-year survival rate of 79% in low HER2/*neu* and negative/nuclear p21 patients, 60% in high HER2/*neu* and negative/nuclear p21 patients, 29% in low HER2/neu and cytoplasmic p21 patients, and 16% in high HER2/*neu* and cytoplasmic p21 patients.**

Conclusions: **The present study, in addition to supporting the mechanisms of p21 regulation derived from laboratory investigation, demonstrates the prognostic importance of phospho-p21 (T145) for the first time and also provides a novel combination of p21 and HER2/*neu* for better stratification of patients' survival than any single clinicopathological or biological marker that may play important diagnostic and therapeutic roles for breast cancer patients.**

INTRODUCTION

Overexpression or amplification of the receptor tyrosine kinase HER2/*neu* (also known as ErbB2) has been noted in ~30% of breast cancer patients and is frequently associated with shorter survival and poor prognostic features, including earlier relapse and increased number of lymph node metastases (1). The underlying mechanisms by which HER2/*neu* promotes tumorigenesis, invasiveness, and metastasis of cancer cells have been extensively studied (2). The phosphatidylinositol-3-OH kinase/Akt pathway is an important HER2/*neu* downstream cascade in preventing cells from undergoing apoptosis and contributing to cell proliferation (3–5). For example, after phosphorylation, the activated Akt can phosphorylate various substrates, such as Bad (6), caspase-9 (7), Forkhead family transcription factors (8, 9), MDM2 (10, 11), and p21$^{Cip1/WAF1}$ (p21; Ref. 12), resulting in either suppression of apoptosis or promotion of cell proliferation. Activation of Akt has also been correlated with poor outcome in breast cancer patients (13).

Among the downstream substrates of Akt, p21 is a critical modulator of cell cycle and cell survival, although its regulation and function have largely remained unclear. p21 was initially considered to be an inhibitor of cell cycle progression and has been shown to suppress tumor formation in xenograft models (14, 15). However, several recent studies have suggested that this protein can also promote cell survival and cell cycle progression (16–18). In addition, elevated p21 protein levels have been observed in various aggressive malignancies, such as glioma and leukemia, and may contribute to chemoresistance (19, 20). The role of p21 in breast cancers has also been controversial in laboratory and clinical studies. It has been shown that HER2/*neu*-overexpressing breast cancer cells can induce chemoresistance through increased expression of p21 (21) and that p21

Received 11/4/03; revised 2/19/04; accepted 3/3/04.
Grant support: Grants RO1 CA58880 and PO1 CA009031 from the National Breast Cancer Research Foundation and Grant CA-16672 from the M. D. Anderson Cancer Center CCSG (M-C. Hung).
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overexpression is associated with poor prognosis in breast cancer patients (22). On the other hand, several studies indicated that p21 expression provides no prognostic information for patients with breast cancer (23, 24). The contradictory effects on tumorigenesis, as well as inconsistent reports about clinical outcomes of p21, could be related to the subcellular localization of this special protein because recent studies have revealed that the cell growth-inhibitory activity of p21 is strongly correlated with its nuclear localization. However, p21 can also localize in the cytoplasm, where it plays an important role in protecting cells from apoptosis (16, 18) and was associated with poor prognosis in breast cancer patients (25).

The cellular localization of p21 has been proposed to be critical for the regulation of p21 function (26), and we recently identified the mechanism by which p21 is phosphorylated by Akt at a consensus threonine residue (threonine 145), which results in cytoplasmic localization and suppression of growth-inhibiting activity (12). Because our previous findings clearly demonstrated the regulation of p21 localization and function of the HER2/*neu*-Akt pathway in a laboratory setting, to further address how this signaling pathway is related to survival and other clinical parameters of breast cancer patients, we analyzed the expression status of p21, HER2/*neu*, and phospho-Akt by immunohistochemical (IHC) staining in 130 breast cancer specimens and compared their expression levels and subcellular localization with clinical outcome. In addition, we used a newly developed antibody against phospho-p21 at threonine 145 [phospho-p21 (T145)] to further validate the results.

MATERIALS AND METHODS

Patients and Tumor Specimens. We obtained 130 archived blocks containing formalin-fixed, paraffin-embedded infiltrating breast carcinoma from the Department of Pathology, Shanghai East Breast Disease Hospital, People's Republic of China. All of the patients were women with nonmetastatic disease who had undergone mastectomy and axillary lymph node dissection between 1988 and 1994. After surgical treatment, the patients were offered adjuvant chemotherapy and/or radiotherapy and hormone therapy, depending on the number of lymph node metastases, status of menopause, and estrogen and/or progesterone receptor positivity. The clinicopathological characteristics of the study population, including age, tumor size, lymph node status, tumor grade, and estrogen receptor/progesterone receptor positivity, were obtained from medical records. The estrogen and progesterone receptor status was unavailable for 20 and 19 tumor specimens, respectively. The stage was assessed by the TNM clinical staging system of the American Joint Committee on Cancer (27). Patients were followed 4–72 months, with a median follow-up of 48 months.

Generation of Anti-Phospho-p21 (T145) Antibody. The polyclonal antibody against phosphorylated human p21 protein was generated by immunization of rabbits with a carrier protein, keyhole limpet hemocyanin, in conjunction with a phosphorylated 11-mer peptide [KRRQT-(PO_3)-SMTDFY] at the terminal region of the p21 sequence encompassing the Akt phosphorylation site (threonine 145). Peptides were synthesized for antibody production by SynPeptide, Inc. (Dubin, CA) and were checked under stringent analytical specifications, which included high performance liquid chromatography, mass spectrometry, and UV analysis. The polyclonal antibody was also generated and affinity-purified by SynPeptide, Inc.

Immunoprecipitation and Immunoblotting. 293T cells were transiently transfected by use of SN liposome (28). After transfection for 36 h, 293T cells were washed with PBS and scraped into RIPA-B buffer. After brief sonication, the cell lysates were centrifuged at 14,300 × *g* for 30 min at 4°C to remove insoluble cell debris. The supernatant was preincubated with protein G-agarose (Roche) for 1 h at 4°C. Flag-tagged p21 was immunoprecipitated overnight with anti-Flag (M2) antibody (Sigma) and protein G-agarose. The immunocomplex was washed four times with RIPA-B buffer, dissolved in sampling buffer, and subjected to SDS-PAGE; the proteins were then transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA in Tris-buffered saline and were incubated with anti-phospho-p21 (T145) antibody (1:1000 diluted in Tris-buffered saline-Tween containing 3% BSA) and then with horseradish peroxidase-conjugated antirabbit secondary antibodies. The immunoblots were visualized by use of the ECL kit (Amersham Pharmacia Biotech).

IHC Staining for p21, Phospho-p21 (T145), HER2/*neu*, and Phospho-Akt. The immunoperoxidase staining method was modified from the avidin-biotin complex technique as described previously (29). In brief, slides (5 μm) were deparaffinized. After antigen retrieval, the slides were digested in 0.05% trypsin. The endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide, and the slides were then treated with 10% normal goat or horse serum for 30 min. After overnight incubation with primary antibodies, including

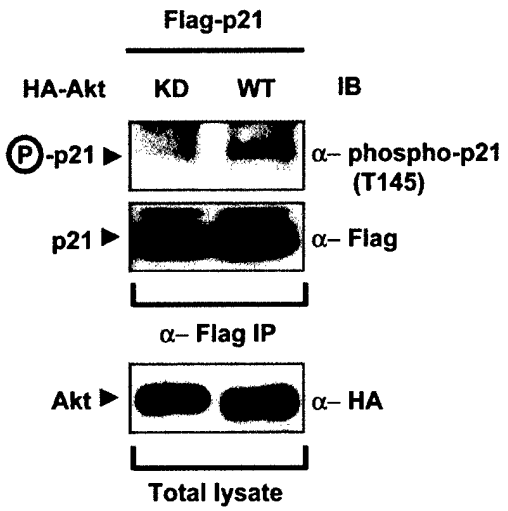

Fig. 1 Detection of p21 (T145) phosphorylation by anti-phospho-p21 (T145) antibody. Flag-tagged p21 and HA-tagged wild-type (*WT*) or kinase-dead Akt (*KD*) were cotransfected transiently into 293T cells. After 36 h of transfection, lysates of cells were subjected to p21 immunoprecipitation (*IP*) with anti-Flag antibody. p21 (T145) phosphorylation by Akt was detected by an anti-phospho-p21 (T145)-specific antibody. Expression levels of Akt (WT) and Akt (KD) were assessed by immunoblotting (*IB*).

(*a*) rabbit polyclonal anti-p21 (c-19; 1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA); (*b*) rabbit polyclonal anti-phospho-p21 (T145) (1:15 dilution; generated by SynPeptide Inc); (*c*) rabbit polyclonal anti-HER2/*neu* (anti-c-erbB-2, 1:300 dilution; DAKO, Carpinteria, CA); and (*d*) rabbit polyclonal anti-phospho-Akt (T308) (1:80 dilution; New England Biolabs Inc.), the slides were incubated with biotinylated secondary antibodies and subsequently incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Antibody detection was performed with the 0.125% aminoethylcarbazole chromogen substrate solution (AEC substrate) from Sigma Chemical Co. After counterstaining with Mayer's hematoxylin (Sigma), the sides were mounted. For negative control, all incubation steps were identical except that PBS was used rather than primary antibody. A previously identified strongly staining tumor tissue section was used as a positive control. The prepared slides were examined by light microscopy.

Fluorescent *in Situ* Hybridization (FISH). The paraffin-embedded tissue sections were baked at 65°C for 2 h and deparaffinized as described previously (29). The FISH assay was performed with the PathVysion HER2 DNA probe Kit (Vysis Inc) according to the manufacturer's recommendation.

Scoring of Immunoreactivity. The immunoreactivity of these antibodies was scored according to the subcellular localization (membrane, nuclear, and/or cytoplasmic), staining intensity (strong, moderate, weak, and faint or slightly above background), and fraction of positive staining. The mean fraction of positive tumor cells was determined in at least nine areas at ×100 or ×200 magnification. p21 immunoreactivity was determined by the percentage of positively stained tumor cells as well as the subcellular localization of staining and was categorized as negative, nuclear, and cytoplasmic. Negative was defined as undetectable cytoplasmic or nuclear staining. Nuclear p21 was defined as the fraction of tumor cells with positive nuclear staining greater than or equal to that of positive cytoplasmic staining. Cytoplasmic p21 was defined as the fraction of cytoplasmic staining greater than that of nuclear staining. HER2/*neu* immunoreactivity was determined by membrane staining and categorized as 0 to 3+. A score of 0 was defined as undetectable staining or membrane staining in <10% of tumor cells, a score of 1+ was defined as faint membrane staining in >10% of tumor cells, a score of 2+ was defined as weak to moderate membrane staining in >10% of tumor cells, and a score of 3+ was defined as intense membrane staining in >10% of tumor cells. The immunoreactivities of phospho-Akt and phospho-p21 (T145) were also ranked into four groups according to the percentage of positively stained tumor cells, including cytoplasmic and nuclear staining: 0, no staining; 1+, <20% cells stained; 2+, 20–49% cells stained; and 3+, >50% cells stained. For analysis, HER2/*neu*, phospho-Akt, and phospho-p21 (T145) expressions was further classified as low (score 0 and 1+) or high (scores 2+ and 3+). The slides were read independently by two investigators without knowledge of the clinical data. If differences between observers occurred, both investigators used a multiheaded microscope to reexamine the slides.

Statistical Analysis. Data on eligible patients were summarized by use of standard descriptive statistics and frequency tabulation. The associations between expression of biomarkers (p21, phospho-p21, HER2/*neu*, and phospho-Akt) and various clinicopathological parameters were assessed by cross-tabulation χ^2 tests. All correlations between each biomarker were analyzed by Kendall's τ-b analysis. The overall survival after surgery was plotted by use of the Kaplan–Meier method. The

Table 1 Clinicopathological and immunohistochemical data for the patients

Characteristic	Patients	
	n	%
Total	130	100
Age (yrs)		
≤48	66	51
>48	64	49
Tumor status		
T_1	66	51
T_2	53	41
T_3	11	8
Lymph node status		
N_0	68	52
N_1	25	19
N_2	32	25
N_3	5	4
Stage		
I	45	35
II	46	35
III	39	30
Tumor grade[a]		
1	28	22
2	43	33
3	59	45
Estrogen receptor		
Negative	68	52
Positive	42	32
Unknown	20	15
Progesterone receptor		
Negative	57	44
Positive	54	41
Unknown	19	15
p21[b]		
Negative	53	41
Nuclear	34	26
Cytoplasmic	43	33
Phospho-p21 (T145)		
Low		
0	64	49
1+	26	20
High		
2+	27	21
3+	13	10
HER2/*neu*		
Low		
0	42	32
1+	42	32
High		
2+	21	16
3+	25	19
Phospho-Akt		
Low		
0	73	56
1+	23	18
High		
2+	14	11
3+	20	15

[a] Tumor grade was classified by WHO criteria (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated).

[b] Nuclear p21, fraction of nuclear staining greater than or equal to fraction of cytoplasmic staining; cytoplasmic p21, fraction of cytoplasmic staining greater than fraction of nuclear staining.

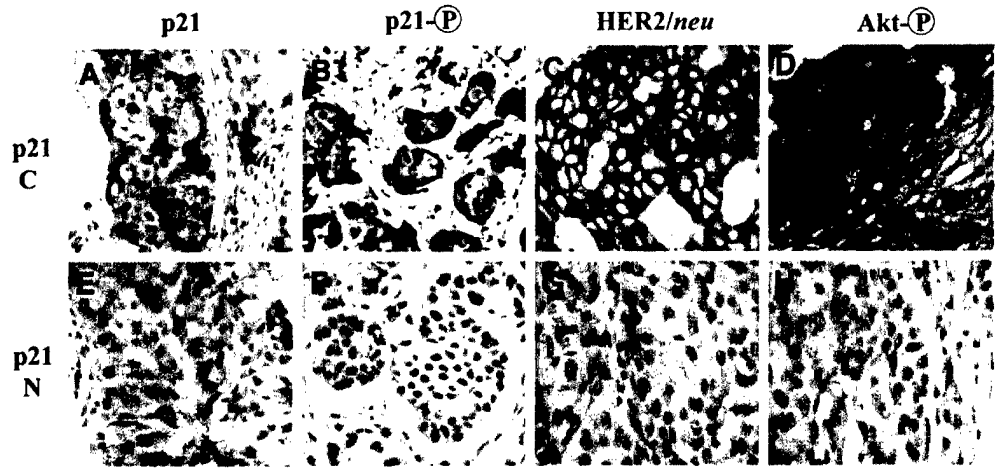

Fig. 2 Representative immunohistochemical pictures of phospho-p21 (T145), HER2/*neu*, and phospho-Akt in cytoplasmic p21 (*C*) or nuclear p21 (*N*) specimens. Tissue sections from patients with cytoplasmic p21 (*A–D*) and nuclear p21 (*E–H*) were stained with specific antibodies against p21 (*A*, cytoplasmic; *E*, nuclear), phospho-p21 (T145) (*B*, overexpression; *F*, negative), HER2/*neu* (*C*, overexpression; *G*, negative), and phsopho-Akt (*D*, overexpression; *H*, negative).

log-rank test was used to analyze differences in survival time. The Cox proportional hazard regression model was used to assess effect of patients' prognostic factors on overall survival. Statistical analysis was performed with SAS 8.0 and S-plus 2000 software. All tests were two-sided, and the level of significance was set at 0.05.

RESULTS

Characterization of Anti-Phospho-p21 (T145) Antibody. Our previous study demonstrated that activated Akt can phosphorylate p21 at threonine 145 and lead to cytoplasmic localization of p21 in HER2/*neu*-overexpressing breast cancer cells (12). To further validate that p21 is phosphorylated on threonine 145 in cancer cells, we developed an anti-phospho-p21 (T145) antibody to recognize this phosphorylation site (see "Materials and Methods"). To characterize the specificity of this antibody, we cotransfected p21 with either wild-type or kinase-dead Akt into 293T cells to determine the specificity of this antibody by a biochemical method. As shown in Fig. 1, phosphorylation of p21 at threonine 145 was detected by this antibody, suggesting that this antibody could recognize phopho-p21 (T145) specifically.

Clinicopathological and IHC Profiles of the Study Population. Patient and tumor characteristics for the entire study population are shown in Table 1. For the 130 patients, the median age was 48 years (range, 26–87 years). No patients had T4 disease or detectable distant metastasis at the time of the surgery. Pathology examination revealed that 118 (91%) of the tumors were infiltrating ductal carcinomas; the remaining 12 (9%) were infiltrating lobular carcinomas. Representative IHC photographs of p21, phospho-p21 (T145), HER2/*neu*, and phospho-Akt are shown in Fig. 2. Positive p21 expression was found in 59% of patients, and the immunoreactivity was in both the cytoplasm and nucleus for the majority of these patients, with 26% mainly in the nucleus and 33% mainly in the cytoplasm. Phospho-p21 (T145) levels were high in 40 (31%) patients, with staining predominantly in the cytoplasm. Phospho-Akt levels were high in 34 (26%) patients, with staining located mainly in the cytoplasm and nucleus. HER2/*neu* levels were high in 46 (35%) patients, with staining mainly in the cell membrane and cytoplasm. To validate the results from the IHC staining, we used FISH to examine randomly selected IHC breast tumors from this cohort. Among HER2/*neu* 3+ and 2+ tissues, we found high degree of correlation, nearly 2+ (87.5%), 3+ (100%), and 0 (100%). Thus, the IHC result is highly consistent with the result from FISH (Table 2 and Fig. 3).

The relationship between various clinicopathological parameters and the biological markers are shown in Table 3. No significant association was noted between the subcellular localization of p21 and age, tumor size, lymph node status, stage, tumor grade, or estrogen and progesterone receptor status. When compared with low expression of phospho-p21, HER2/*neu*, and phospho-Akt, overexpression of these biological markers was also not associated with any clinicopathological parameter of breast cancer patients.

Cytoplasmic p21 Is Highly Correlated with Overexpression of Phospho-p21 (T145), Which Is Located Primarily in the Cytoplasm. To further study the correlation between phospho-p21 (T145) and overall survival of breast cancer patients,

Table 2 Comparison of immunohistochemical staining and fluorescence *in situ* hybridization

	HER2/*neu* IHC			
HER2/*neu* probe[a] FISH[b]	3+	1+	0	Total
Amplified	5	7	0	12
Nonamplified	0	1	5	6
Total	5	8	5	18

[a] Pearson's χ^2 test (SPSS): $P = 0.95$ ($P > 0.05$).
[b] FISH, fluorescence *in situ* hybridization; IHC, immunohistochemical staining.

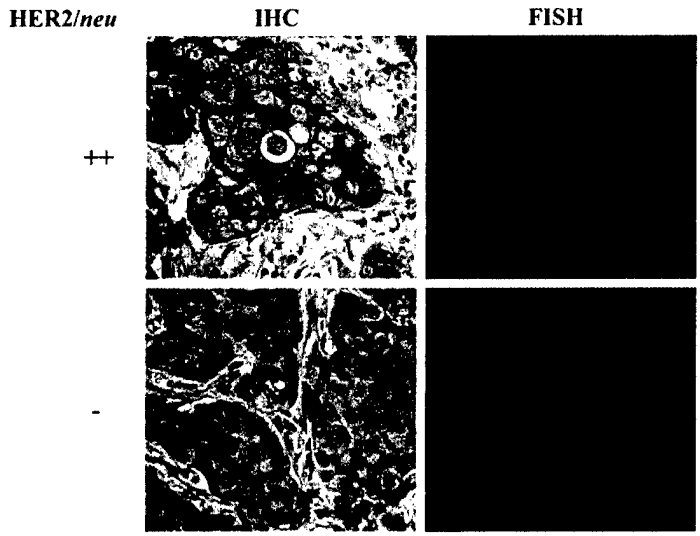

Fig. 3 HER2/*neu* expression and amplification detected by immunohistochemical staining (*IHC*) and fluorescence *in situ* hybridization (*FISH*), respectively. IHC negative (−) is related to FISH nonamplified (−); IHC high positive (++) is correlated to FISH amplified (++). For the FISH assay, *green* is chromosome 17 centromere; and *red* is HER2/*neu*.

we examined surgical specimens from breast cancer patients immunohistochemically, using this antibody. The subcellular location of positive staining was predominantly in the cytoplasm (Fig. 2*B*). Nuclear staining could be detected in only 2 of the 66 specimens with cytoplasmic staining and was very weak. In addition, the expression of phospho-p21 (T145) was highly correlated with the subcellular localization of p21 (Table 4). When p21 was localized mainly in the cytoplasm, phospho-p21 (T145) was likely to be highly expressed (see also Fig. 2, *A* and *B*). In contrast, when p21 was located mainly in the nucleus, phospho-p21 (T145) expression tended to be low or negative [see Fig. 2, *E* and *F*; correlation coefficient (r) = 0.33; P = 0.001].

Table 3 Association of p21, phospho-p21, HER2/*neu*, and phospho-Akt with clinicopathological parameters in 130 breast cancer patients
$P > 0.05$ for all associations between biomarkers and clinicopathological variables analyzed by cross-tabulation (χ^2 test).

	No. of patients (%)			
Parameters	Cytoplasmic p21[a]	High phospho-p21 (2+/3+)	High HER2/*neu* (2+/3+)	High phospho-Akt (2+/3+)
Total (n)	43	40	46	34
Age (yrs)				
≤48	20 (15.4%)	18 (13.8%)	25 (19.2%)	16 (12.3%)
>48	23 (17.7%)	22 (16.9%)	21 (16.2%)	18 (13.8%)
Tumor size				
T_1	17 (13.1%)	17 (13.1%)	19 (14.6%)	18 (13.8%)
T_2 and T_3	26 (20.0%)	23 (17.7%)	27 (20.8%)	16 (12.3%)
Lymph node				
Negative	18 (13.8%)	17 (13.1%)	21 (16.2%)	16 (12.3%)
Positive	25 (19.2%)	23 (17.7%)	25 (19.2%)	18 (13.8%)
Stage				
I	9 (6.9%)	12 (9.2%)	14 (10.8%)	14 (10.8%)
II	17 (13.1%)	12 (9.2%)	13 (10.0%)	6 (4.6%)
III	17 (13.1%)	16 (12.3%)	19 (14.6%)	14 (10.8%)
Tumor grade				
1	6 (4.6%)	7 (5.4%)	6 (4.6%)	6 (4.6%)
2	12 (9.2%)	13 (10.0%)	14 (10.8%)	13 (10.0%)
3	25 (19.2%)	20 (15.4%)	26 (20.0%)	15 (11.5%)
Estrogen receptor[b]				
Negative	16 (14.5%)	18 (16.4%)	19 (17.3%)	20 (18.2%)
Positive	15 (13.6%)	15 (13.6%)	17 (15.5%)	9 (8.2%)
Progesterone receptor[c]				
Negative	15 (13.5%)	20 (18.2%)	15 (13.5%)	13 (11.7%)
Positive	16 (14.4%)	9 (8.2%)	21 (18.9%)	16 (14.4%)

[a] p21 status was divided into three groups: negative, nuclear, and cytoplasmic.
[b] Data from 20 patients are missing.
[c] Data from 19 patients are missing.

Table 4 Correlation between subcellular localization of p21 and immunoreactivity of phospho-p21 (T145)
$r = 0.33$; $P = 0.001$ (Kendall's τ -b analysis).

Phospho-p21 staining	p21 subcellular localization, *n* (%)		
	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total[a] ($n = 77$)
0	16 (21%)	6 (8%)	22 (29%)
1+	8 (10%)	10 (13%)	18 (23%)
2+	6 (8%)	18 (23%)	24 (31%)
3+	4 (5%)	9 (12%)	13 (17%)

[a] Fifty-three patients with negative staining for p21 were excluded.

Table 5 Correlation between immunoreactivity of HER2/*neu* and phospho-Akt
$r = 0.45$; $P < 0.0001$.

Phospho-Akt staining	HER2/*neu* staining, *n* (%)				
	0 ($n = 42$)	1+ ($n = 42$)	2+ ($n = 21$)	3+ ($n = 25$)	Total ($n = 130$)
0	38 (29%)	20 (15%)	8 (6%)	7 (5%)	73 (56%)
1+	3 (2%)	10 (8%)	6 (4%)	4 (3%)	23 (18%)
2+	1 (1%)	7 (5%)	2 (2%)	4 (3%)	14 (11%)
3+	0 (0%)	5 (4%)	5 (4%)	10 (8%)	20 (15%)

Cytoplasmic Localization of p21 and Overexpression of Phospho-p21 (T145) Are Associated with Overexpression of HER2/*neu* and Phospho-Akt. Because we previously demonstrated that activated Akt phosphorylates p21 and determined its subcellular localization in HER2/*neu*-overexpressing cells (12), we examined the correlations among these molecules in this pathway. As shown in Table 5, the level of phospho-Akt expression was strongly correlated with the level of HER/*neu* expression, supporting the previous report that HER2/*neu* overexpression activates Akt by phosphorylation of Akt (12). The association between the levels of HER2/*neu* or phospho-Akt expression and the cellular localization of p21 was also significant (Tables 6 and 7), indicating that when HER2/*neu* or phospho-Akt was highly expressed, p21 was localized mainly in the cytoplasm (see Fig. 2, *A*, *C*, and *D*). In contrast, when the levels of HER2/*neu* or phospho-Akt expression were low or negative, p21 tended to be negative or localized mainly in the nucleus (see Fig. 2, *E*, *G*, and *H*). Furthermore, the levels of HER2/*neu* and phospho-Akt expression were positively associated with the levels of phospho-p21 (T145) expression ($r = 0.49$, $P < 0.001$ for HER2/*neu*; $r = 0.52$, $P < 0.001$ for phospho-Akt). All of these data consistently support the hypothesis that overexpression of HER2/*neu* can activate/phosphorylate Akt, which in turn phosphorylates p21 at threonine 145 and leads to cytoplasmic localization of p21.

Cytoplasmic Localization of p21 and Overexpression of Phospho-p21 (T145) Are Associated with Poor Patient Survival. We next sought to clinically determine whether subcellular localization of p21 and the levels of phospho-p21 (T145)

Table 6 Correlation between subcellular localization of p21 and HER2/*neu* immunoreactivity
$r = 0.37$; $P = 0.0003$.

HER2/*neu* staining	p21 subcellular localization, *n* (%)			
	Negative ($n = 53$)	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total ($n = 130$)
0	28 (22%)	10 (8%)	4 (3%)	42 (32%)
1+	15 (12%)	13 (10%)	14 (11%)	42 (32%)
2+	5 (4%)	5 (4%)	11 (9%)	21 (16%)
3+	5 (4%)	6 (5%)	14 (11%)	25 (19%)

Table 7 Correlation between subcellular localization of p21 and immunoreactivity of phospho-Akt
$r = 0.32$; $P = 0.0011$.

Phospho-Akt staining	p21 subcellular localization, *n* (%)			
	Negative ($n = 53$)	Nuclear ($n = 34$)	Cytoplasmic ($n = 43$)	Total ($n = 130$)
0	38 (29%)	21 (16%)	14 (11%)	73 (56%)
1+	10 (8%)	3 (2%)	10 (8%)	23 (18%)
2+	4 (3%)	3 (2%)	7 (5%)	14 (11%)
3+	1 (1%)	7 (5%)	12 (9%)	20 (15%)

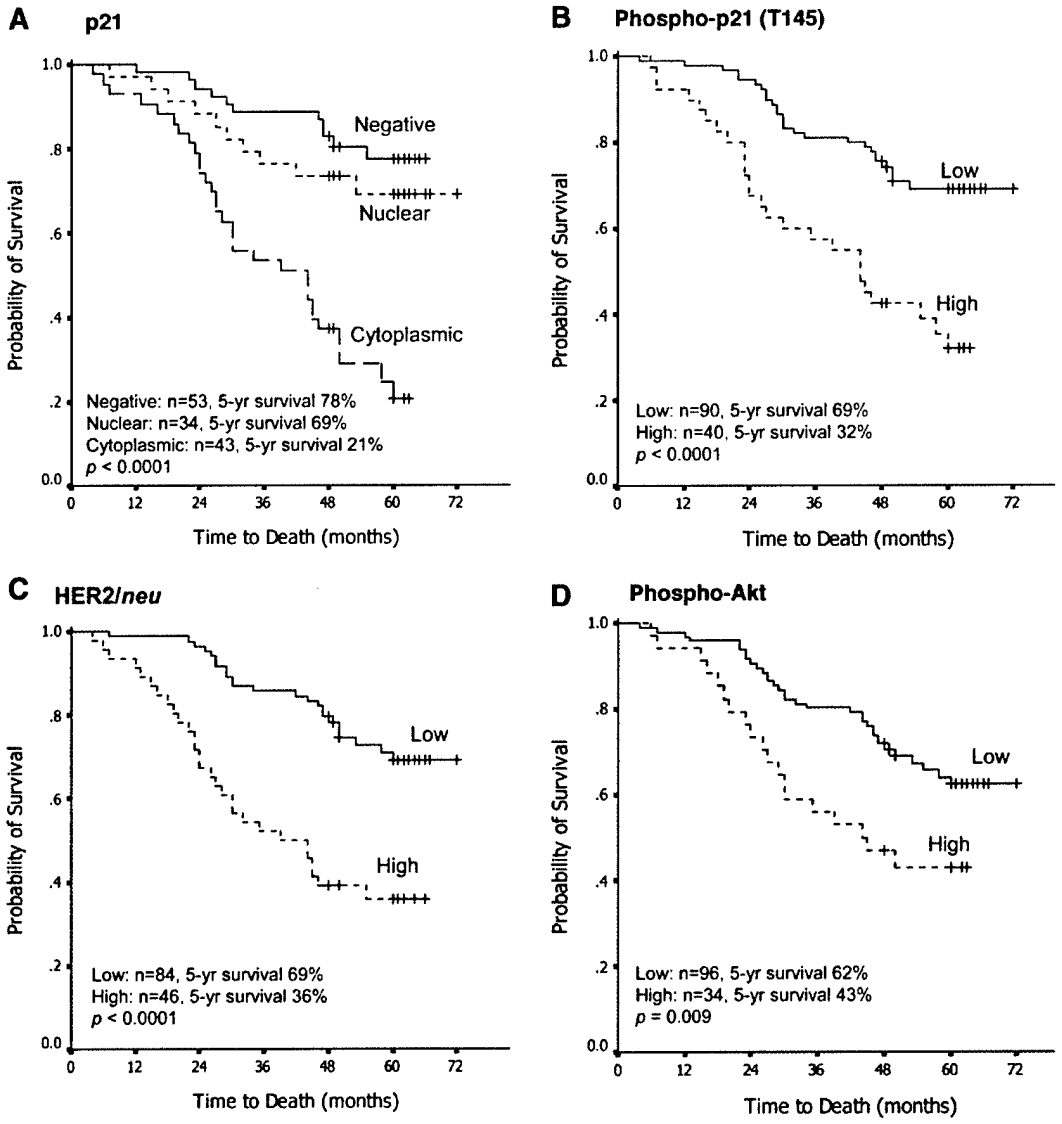

Fig. 4 Kaplan-Meier analyses of overall survival of breast cancer patients. Survival curves are stratified by expression status of p21 (*A*), phospho-p21 (T145) (*B*), HER2/*neu* (*C*), and phospho-Akt (*D*). Differences in survival distributions were evaluated by log-rank tests.

expression affected patient survival. We found that the overall survival of patients with p21 localized in the cytoplasm was much worse than that of patients in whom p21 was negative or localized in the nucleus ($P < 0.0001$; Fig. 4*A*). However, the difference in overall survival between patients negative for p21 and for nuclear p21 was not significant ($P = 0.32$). Consistent with cytoplasmic localization of p21, high levels of phospho-p21 (T145) were also associated with poor patient survival compared with low phospho-p21 (T145) levels (Fig. 4*B*). In addition, poor overall survival was noted in patients with overexpression of HER2/*neu* and phospho-Akt (Fig. 4, *C* and *D*). The effects of p21 subcellular localization and phospho-p21 (T145), HER2/*neu*, and phospho-Akt status on disease-free survival revealed trends similar to those for overall survival (data not shown).

Combined Subcellular Localization of p21 and Expression Status of HER2/*neu* Provide a Better Prognostic Factor. To determine the prognostic importance of clinicopathological parameters and the HER2/*neu*-Akt-p21 pathway members in breast cancer, we performed Cox proportional hazard regression analyses on 130 breast cancer patients. Univariate analysis revealed that large tumor size; advanced nodal status; advanced TNM stage; overexpression of HER2/*neu*, phospho-Akt, and phospho-p21 (T145); and cytoplasmic localization of p21 were associated with a poor outcome (Table 8*A*). In multivariate analysis, only large tumor size, advanced nodal status, overex-

Table 8 Cox's proportional hazard model analysis of prognostic factors in breast cancer patients

Variable	Parameter estimate	SE	*P*	Relative risk
A. Univariate analysis				
Age (> 48 *vs.* ≤48 yrs)	0.41	0.28	0.14	1.50
Tumor (II *vs.* I)	0.91	0.32	0.004	2.49
Tumor (III *vs.* I)	1.95	0.42	<0.0001	7.03
Node (N_1 *vs.* N_0)	0.68	0.40	0.09	1.97
Node (N_2 or N_3 *vs.* N_0)	0.80	0.37	0.033	2.22
Stage (II or III *vs.* I)	1.17	0.37	0.001	3.23
Grade (2 or 3 *vs.* 1)	0.69	0.41	0.09	1.98
HER2/*neu* (high *vs.* low)	1.31	0.28	<0.0001	3.72
Phospho-Akt (high *vs.* low)	0.77	0.29	0.008	2.16
Phospho-p21 (T145) (high *vs.* low)	1.22	0.28	<0.0001	3.39
p21 (cytoplasmic *vs.* others)	1.55	0.29	<0.0001	4.73
HER2/*neu* (low) + p21 (cytoplasmic)[a]	1.35	0.43	0.0018	3.85
HER2/*neu* (high) + p21 (neg.[b]/nuclear)[a]	1.04	0.44	0.018	2.83
HER2/*neu* (high) + p21 (cytoplasmic)[a]	2.32	0.37	<0.0001	10.18
B. Multivariate analysis				
Tumor status (T_2 *vs.* T_1)	0.42	0.33	0.21	1.52
Tumor status (T_3 *vs.* T_1)	2.17	0.47	<0.0001	8.77
Node (N_1 *vs.* N_0)	0.56	0.41	0.17	1.75
Node (N_2 or N_3 *vs.* N_0)	0.70	0.39	0.071	2.01
HER2/*neu* (high *vs.* low)	1.09	0.31	0.001	2.97
p21 (cytoplasmic *vs.* others)	1.31	0.31	<0.0001	3.71
HER2/*neu* (low) + p21 (cytoplasmic)[a]	1.37	0.44	0.002	3.92
HER2/*neu* (high) + p21 (neg./nuclear)[a]	1.15	0.45	0.011	3.15
	2.41	0.39	<0.0001	11.08

[a] The combination of HER2/*neu* (low) and p21 (negative/nuclear) is used as the baseline. The relative risk of the combined HER2/*neu* (low) and p21 (cytoplasmic) against baseline is 3.85 by univariate analysis, 3.92 by multivariate analysis.
[b] neg., negative.

pression of HER2/*neu*, and cytoplasmic localization of p21 were significant and independent prognostic factors in this study (Table 8*B*). To better predict the prognosis of breast cancer patients, we combined the expression status of HER2/*neu* and subcellular localization of p21, creating a new prognostic factor. To simplify the classification, patients negative for p21 and nuclear p21 were combined into one group (negative/nuclear p21) because their survival rates were not significantly different. When patients' overall survival was stratified by this new combined factor, the 5-year survival rates were, respectively, 79, 60, 29, and 16% for the four groups: (*a*) patients with low HER2/*neu* and negative/nuclear p21; (*b*) patients with high HER2/*neu* and negative/nuclear p21; (*c*) patients with low HER2/*neu* and cytoplasmic p21; and (*d*) patients with high HER2/*neu* and cytoplasmic p21 ($P < 0.001$; Fig. 5*A*). This new factor was found to be a more powerful predictor of patient survival than any individual clinicopathological or biological marker in this study by Cox regression model and Kaplan-Meier survival analyses (see also Table 8 and Fig. 4), with a relative risk by multivariate analysis of 11.08 ($P < 0.0001$) for patients with overexpressed HER2/*neu* and cytoplasmic p21. Furthermore, the predictive power and accuracy of this new prognostic factor could be comparable to TNM staging, the "gold standard" prognostic determinant of breast cancer (Fig. 5*B*).

DISCUSSION

The present study examined the expression status of the key members of the HER2/*neu*-Akt-p21 pathway in breast cancers as well as their relationship with clinicopathological parameters and patient survival. We demonstrated that the cytoplasmic localization of p21 is highly correlated with overexpression of phospho-p21 (T145). Both cytoplasmic p21 and overexpression of phospho-p21 (T145) are associated with high expression levels of HER2/*neu* and phospho-Akt, and all of these biological conditions are associated with poor survival of breast cancer patients. In addition, by the multivariate Cox proportional hazard model, overexpression of HER2/*neu* and cytoplasmic p21 were found to be significant and independent factors in predicting outcome. Further stratification of patients' survival by combined HER2/*neu* and p21 status provides a more accurate prediction than any individual clinicopathological and biological factor. The predictive power of this new combined factor could be comparable to that of TNM staging.

The role of p21 in the prognosis of breast cancer patients has been controversial. Several studies demonstrated that by using a monoclonal antibody (clone 4D10, mouse IgG1 subtype; Novocastra, Newcastle upon Tyne, United Kingdom), the expression of p21 was predominantly in the nucleus and provided no prognostic information (23, 24). However, using another monoclonal antibody (Ab-1; Calbiochem, Cambridge, MA), one study revealed that p21 immunoreactivity was both cytoplasmic and nuclear in the majority of breast cancers and that cytoplasmic p21, rather than the total p21 level, was associated with poor survival (25). In our study, two antibodies that recognized different forms of p21 were used. With anti-p21 (c-19; Santa Cruz Biotechnology), the immunoreactivity was both cytoplas-

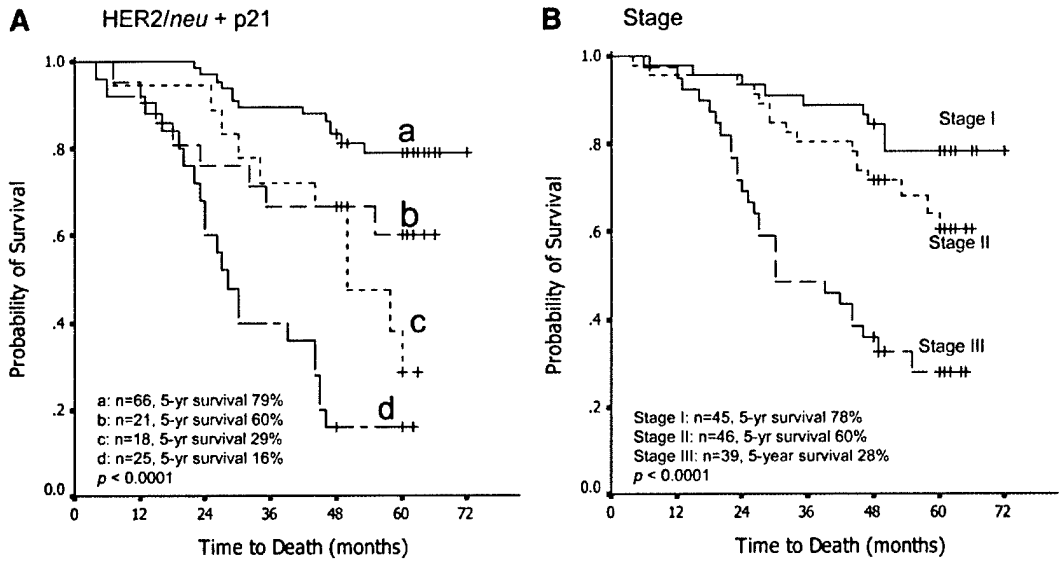

Fig. 5 Kaplan-Meier analyses of overall survival of breast cancer patients. Survival curves are stratified by a combination of expression status of HER2/*neu* and subcellular localization of p21 (*A*; *line a*, low HER2/*neu* and negative/nuclear p21; *line b*, high HER2/*neu* and negative/nuclear p21; *line c*, low HER2/*neu* and cytoplasmic p21; *line d*, high HER2/*neu* and cytoplasmic p21) and TNM stage (*B*). Differences in survival distributions were evaluated by log-rank tests.

mic and nuclear. With anti-phospho-p21 (T145), the immunoreactivity was primarily in the cytoplasm. Both antibodies consistently showed that p21, particularly the phosphorylated form, can be localized in the cytoplasm and that cytoplasmic localization of p21 was associated with a poor outcome for breast cancer patients. On the other hand, nuclear p21 was more closely associated with low levels of HER2/*neu* and phospho-Akt, and the overall survival of patients with nuclear p21 was not significantly different from that of those negative for p21. Our results suggest that the previous controversy over clinical results might be caused by the different antibodies used in IHC staining and different interpretation of subcellular localization of p21.

Many laboratory findings suggest that p21 might play dual roles in regulating cell cycle progression and apoptosis by changing its subcellular localization. Our data demonstrate that cytoplasmic p21 has a prognostic implication different from that of nuclear p21 in breast cancer patients. However, the molecular mechanisms by which p21 changes its subcellular localization remained elusive until we reported that Akt may play a pivotal role in p21 localization (12). In that study, we showed that activated Akt physically associates with and phosphorylates p21 at threonine 145, resulting in cytoplasmic localization of p21. Blocking of the Akt pathway with an Akt (KD) mutant restores the nuclear localization and cell-growth inhibition of p21. In the present study, we consolidate these findings in a clinical setting by showing that overexpression of activated/phosphorylated Akt is highly correlated with overexpression of phospho-p21 (T145) and cytoplasmic localization of p21 and that phospho-p21 (T145), when detected, is primarily localized in the cytoplasm. Our results also indicate that the Akt-p21 pathway plays an important role in the prognosis of breast cancer patients because high levels of phospho-Akt and phospho-p21 (T145) and cytoplasmic localization of p21 are associated with poor patient survival.

Although we clearly showed that activated Akt induces cytoplasmic localization of p21 and correlates with shorter survival of patients, the clinical sequence by which cytoplasmic p21 leads to poor prognosis remains unclear. Using a multivariate Cox proportional hazard model and analyses of the correlation with clinicopathological parameters, we found that cytoplasmic p21 is an independent prognostic factor that correlated with no clinicopathological parameters, such as large tumor size, advanced lymph node metastases, or poor tumor grade in this study. However, recent studies revealed that breast cancer cells and leukemia cells can induce resistance to chemotherapeutic drugs through overexpression of p21 (20, 21) and that activation of Akt can lead to cytoplasmic localization and stabilization of p21 (12, 30). We therefore hypothesize that after phosphorylation by activated Akt, p21 can increase its expression levels, translocate into the cytoplasm, induce the phenotype of chemoresistance, and finally lead to poor survival of breast cancer patients irrespective of tumor size, grade, and lymph node status.

The relationship between HER2/*neu* and the Akt-p21 pathway has rarely been addressed. In this study, we found that cytoplasmic localization of p21 and overexpression of phospho-p21 (T145) and phospho-Akt were highly associated with overexpression of HER2/*neu*, in concordance with our previous finding that the Akt pathway is required for HER2/*neu*-mediated cell proliferation (12). However, our patients' data also implied that overexpression of HER2/*neu* may not be the only mechanism to induce Akt activation and p21 phosphorylation/cytoplasmic localization in breast cancer patients because the mul-

tivariate Cox proportional hazard model revealed that HER2/*neu* overexpression and p21 cytoplasmic localization are significant but independent prognostic factors, suggesting that, in addition to HER2/*neu*, other pathways could activate Akt or phosphorylate p21 and cause p21 to localize in the cytoplasm. Furthermore, overexpression of HER2/*neu* can reduce patient survival through many pathways other than Akt-p21.

The advance of laboratory studies has provided many biological markers to predict the prognosis of patients and provide targets for therapy. The accumulated results of studies also indicate that, with appropriate selection, multiple markers might be more informative than any single marker for the prediction of clinical outcome in breast cancer patients (31). In this study, in addition to consolidating the mechanisms of p21 regulation through the activation of the HER2/Akt pathway, we also demonstrated that cytoplasmic p21 was an independent prognostic factor in breast cancer patients. A novel combination of subcellular localization of p21 and expression status of HER2/*neu* clearly stratified the patients into four distinctly different survival groups. Among them, the 5-year survival rate of patients with low HER2/*neu* and negative/nuclear p21 was 79%, in contrast to only 16% in those patients with high HER2/*neu* and cytoplasmic p21. This novel combination not only provides a better prognostic prediction than any individual clinicopathological or biological marker, it also indicates that targeting only one molecule, such as HER2/*neu*, could be insufficient. Novel therapeutic agents that target phosphorylation/cytoplasmic localization of p21 may also contribute to optimal treatment of breast cancer patients.

ACKNOWLEDGEMENTS

We thank Dr. Stephanie Miller for editing this article.

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E1A-mediated sensitization to anti-cancer drug-induced apoptosis in human breast cancer (Abstract).

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Most chemotherapeutic drugs kill cancer cells by inducing apoptosis, and a deficiency in apoptosis often confers drug resistance. Therefore, a novel strategy for combating cancer is to restore the sensitivity of cancer cells to apoptosis. By studying of stable cells in vitro and systemic gene therapy in othortopic breast cancer model in animal model in vivo, we showed that introducing adenovirus type 5 early region 1A (E1A) into tumor cells resulted in sensitization to paclitaxel (Taxol)-induced apoptosis and enhancement of Taxol antitumor activity. Molecular analysis indicated that this beneficial anti-tumor effect was achieved, at least partly, by E1A-mediated down regulating Akt activity and up regulating p38 activity in breast cancer cells. The same mechanism was observed using additional anti-cancer drugs, such as doxorubicin (Adriamycin), cis-platin, methotrexate, and gemcitabine. Further genetic analysis reveiled that disrupting Rb pathway by E1A was required for chemosensitization, whereas disrupting E1A's ability to bind with p300 did not abrogate E1A-mediated chemosensitization. Thus, our results support that integration of pro- and anti-apoptotic signalings by E1A contributed to E1A-mediated sensitization to chemotherapeutic drug-induced apoptosis.

This work was supported by Grant RO1-CA58880 from the National Cancer Institutes of Health (to M.-C. H.) and the U.S. Army Medical Research and Material Command under DAMD17-01-1-0300 (to Y. L.).